EVIDENCE APPENDIX

PART I

EXHIBIT A

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 6. Number 2. 1990
Mary Ann Liebert. Inc.. Publishers

Selective Killing of HIV-Infected Cells by Anti-gp120 Immunotoxins

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ABSTRACT

Either ricin A chain (RAC) or *Pseudomonas* exotoxin (PE) was conjugated with a murine monoclonal antibody (0.5β) directed against an external envelope glycoprotein (gp120) of human immunodeficiency virus (HIV). Effects of the immunotoxins produced against infected cells were evaluated. Selective inhibition of the proliferation and killing of chronically HIV infected cells were observed in the presence of the immunotoxins. To determine the feasibility of the immunotoxins against the infected cells in seropositive subjects, we attempted to detect gp120-bearing cells in peripheral blood mononuclear cells (PBM) by cytofluorography. Cells in the monocyte/macrophage region of 2 of 10 PBM samples from HIV-infected individuals were found to react with 0.5β (18.1% and 12.8%). Furthermore, the cell population which was reactive with 0.5β was also susceptible to RAC conjugated with 0.5β. These results suggest that the strategy of using anti-gp120 immunotoxin to eliminate HIV-infected cells may be feasible in infected individuals.

INTRODUCTION

HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 (HIV-1) is a human retrovirus which is the etiologic agent for the acquired immunodeficiency syndrome (AIDS) and related disorders.[1,2] Some HIV-infected T cells and macrophages appear to be resistant to the cytopathic effect of the virus[3-5] and continue to produce a large quantity of virions in vivo.[6,7]

Immune responses against HIV-1 such as antibody-dependent cellular cytotoxicity (ADCC), cytotoxic T lymphocyte (CTL), and antibody-dependent complement-mediated cytolysis (ACC) have been considered to be important for eliminating HIV-infected cells in the body. HIV-specific CTL and ADCC were demonstrated in seropositive cases.[7-9] On the other hand, no ACC activity was detected in many antibody-positive patients' sera.[10] HIV-neutralizing antibodies were also detected in seropositive individuals[11,12] and in some cases, were associated with better clinical courses.[13,14] In

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experimental animals, neutralizing antibodies,[15-17] CTL[18] ADCC[19] and ACC[10] against HIV were inducible by immunization of purified or recombinant external envelope glycoprotein (gp120) of HIV. However, it still remains to be elucidated whether an HIV vaccination with already infected people are effective or not.[20]

As an alternative strategy we investigated the possibilities of eliminating the HIV-infected cells by a passive immunotherapy using immunotoxins directed against gp120 of HIV. Immunotoxins are artificially constructed hybrid molecules in which a toxic compound attaches to an antibody that specifically recognizes target cells.[21] We have recently reported a monoclonal antibody (0.5β) that was reactive against gp120 of HIV and was capable of neutralizing cell-free and cell-associated virus infection in an isolate-specific fashion.[22] The epitope recognized by 0.5β was mapped to a 24 amino acid segment which was within a highly variable region of gp120. We have constructed anti-gp120 immunotoxins by conjugating 0.5β with toxins. We now report that anti-gp120 immunotoxins selectively eliminate HIV-infected cells not only in chronically HIV-infected cell lines, but also in freshly isolated peripheral blood mononuclear cell (PBM) in vitro.

MATERIALS AND METHODS

Cells and viruses

Uninfected H9 and CEM cells, H9 cells chronically infected with human T-lymphotropic virus type IIIB (H9/IIIB), and CEM cells chronically infected with lymphadenopathy-associated virus type-1 (CEM/LAV-1) have been described.[1,2] H9 and H9/IIIB cells and CEM/LAV-1 cells were kindly provided by Drs. R.C. Gallo and L. Montagnier, respectively. A CEM/LAV-1 subline, over 80% of them stably expressed gp120 was used for the study. Cells were maintained in RPMI 1640 medium (GIBCO) with 20% fetal calf serum (FCS) and antibiotics (complete medium).

Antibody and immunotoxins

A type-specific neutralizing monoclonal antibody (0.5β) against the exterior envelope protein (gp120) of HIV[22] was used to prepare anti-gp120 immunotoxins. Ion-exchange chromatography was used to purify 0.5β. Purified ricin A chain (RAC) was purchased from E-Y Laboratories (San Mateo, CA). Next, 0.5β antibody was cross-linked to RAC using the heterobifunctional reagent SPDP [N-succinimidyl 3-(2-pyridyldithio) propionate, (SPDP) Pharmacia] as described by Krolick et al.[23] Briefly, purified 0.5β in phosphate-buffered saline (PBS) (0.01 *M* sodium phosphate, pH 7.4, containing 0.15 *M* NaCl) were treated with 15-fold molar excess of SPDP for 30 min at room temperature to introduce 2-pyridil disulfide groups into the IgG molecule. The modified and dialyzed proteins were then mixed with three-fold molar excess of the purified, reduced RAC in PBS and incubated at 4°C for 15 h. The 0.5β-RAC conjugates (RAC-β) were separated from unbound RAC by gel filtration on a Sephacryl S-200 column. A high-performance liquid chromatography (HPLC) analysis revealed that the purified immunotoxin contained one ricin moiety per antibody. Slight contamination of free antibody was detected, but no free ricin was detected in the preparation (data not shown). The 0.5β antibody was also conjugated with *Pseudomonas* exotoxin A (PE) (Seikagaku Kogyo, Tokyo, Japan) by a method similar to that described for RAC-β except that PE was first conjugated with SPDP, reduced, and conjugated with 0.5β pretreated with SPDP. The 0.5β-PE conjugates (PE-β) were purified as described above.

Cytotoxicity assay for the immunotoxins against infected cell line

Cytotoxic activity of the immunotoxins was assessed by a cell killing assay. A half million of H9/IIIB, H9, CEM/LAV-1, or CEM cells were cultured in the presence or absence of various concentrations of the immunotoxins and incubated at 37°C in 5% CO_2-containing humidified air. Viable cell

numbers were counted every 12 h in a hemocytometer under the microscope by trypan blue dye exclusion method. In the same experiment, H9/IIIB cells, which were cultured in the presence or absence of various concentrations of the immunotoxins, were collected every 12 h, washed twice with PBS, and fixed on glass slides. HIV p24 antigen-positive cells were detected by anti-p24 monoclonal antibody as previously described.[24]

Peptide and ELISA

The 33 amino acid peptide ABJ709 (Pro-Asn-Asn-Asn-Thr-Arg- Lys-Ser-Ile-Arg-Ile-Gln-Arg-Gly-Pro-Gly-Arg-Ala-Phe-Val-Thr-Ile-Gly-Lys-Ile-Gly-Asn-Met-Arg-Gln-Ala-His-Cys) was synthesized by the solid-phase method on an automated peptide synthesizer (Applied Biosystems 430A) by Applied Biosystems Japan. Serum samples were obtained from asymptomatic HIV carriers and patients with AIDS-related complex (ARC), human T-cell leukemia virus type I (HTLV-I) carriers, and seronegative controls. Binding of patients' sera against the peptide was assessed by ELISA. Flexible assay plates (Falcon 3912, Becton Dickinson) were coated with or without ABJ709 peptide at a concentration of 3 μg/ml in PBS (pH 7.2) overnight. After blocking with 2% bovine serum albumin (BSA) containing PBS and washing with PBS, 100 μl of 1:2000 diluted serum samples were reacted for 2 h followed by incubation with alkaline phosphatase-(ALP) conjugated anti-human IgG and substrate (both from Sigma).

An inhibition ELISA that detected antibodies in the patients' serum that competed in binding of 0.5β to ABJ 709 was developed as follows. Microtiter plates were coated with 3 μg/ml of ABJ709 peptide. After blocking with 2% BSA-PBS, aliquots (50 μl) of serially diluted serum samples were added with 50 μl of 0.5β antibody (3 μg/ml). The competition in the binding of 0.5β with human serum samples was detected by ALP-conjugated anti-mouse IgG and substrate.

Flow cytometric analysis

Reactivities of 0.5β antibody against fresh peripheral blood mononuclear cells (PBM) from seropositive individuals were analyzed by flow cytometry. PBM cells were obtained by Ficoll-Conray density gradient centrifugation and incubated 1 h with normal human IgG (200 μg/ml) to saturate Fc receptors on the cell membrane. PBM cells were then pelleted and reacted with 0.5β or control IgG1 antibody followed by staining with fluorescein isothiocyanate (FITC) -conjugated $F(ab')_2$ fragment of anti-mouse IgG antibody (Sigma), and analyzed by laser flow cytometry (FACSTAR, Becton Dickinson) as previously described.[22]

Cytotoxicity assay for the immunotoxin against fresh PBM cells

PBM cells (10^6) from an ARC patient were resuspended in the complete medium containing normal human IgG (200 μg/ml) and cultured in the presence of the following reagents: samples 1 and 2; MOPC 21 (10 μg/ml), sample 3; 0.5β (10 μg/ml), sample 4; RAC-β (1 μg/ml). After 36 h, cultivation at 37°C in 5% CO_2 containing incubator, cells in each sample were collected, pelleted, and reacted with either MOPC 21 (sample 1) or 0.5β (samples 2,3,4). The cells were further stained with FITC-conjugated $F(ab')_2$ fragment of anti-mouse IgG antibody (Sigma) and analyzed by laser flow cytometry as described.

RESULTS

Ricin A chain (RAC) enzyme binds to the 60s ribosomal subunit at or near the elongation factor-2 binding site, causing inhibition of protein synthesis and eventually, cell death. One molecule of RAC in the cytosol is sufficient to kill a cell.[21] The 0.5β antibody was conjugated with RAC (RAC-β) and the toxic activity against H9/IIIB cells or uninfected H9 cells by RAC-β was tested. When H9/IIIB

cells were exposed to RAC-β, the growth of the H9/IIIB cells was inhibited within a day in culture followed by cell death (Fig. 1). By contrast, when uninfected H9 cells were cultured in the presence of RAC-β, no inhibition in growth was detected except for a slight suppression in growth on the second day in culture at the highest concentration of RAC-β. The effect of RAC-β on the expression of HIV p24 *gag* antigen in H9/IIIB cells was also studied by indirect immunofluorescence. The percentages of p24-positive cells decreased with time in culture when the cells were cultured with RAC-β (Fig. 2). The result suggests that RAC-β selectively killed p24-positive cells in the H9/IIIB cell line. A small fraction of p24-positive cells that survived in the presence of RAC-β could be explained by the fact that these cells were the HIV-infected but low or nonproducer cells of the virus or the cells infected with defective viruses. However, these surviving fractions, were also susceptible to RAC-β, and could not be maintained longer than 10 days in the presence of RAC-β.

Next, 0.5β was conjugated with *Pseudomonas* exotoxin A (PE-β). PE is also an inhibitor of ribosomal protein synthesis.[21] PE-β inhibited the proliferation of lymphadenopathy-associated virus type 1-(LAV-1) infected subline of CEM cells (Fig. 3). No inhibition in growth was observed when uninfected CEM cells were exposed to PE-β.

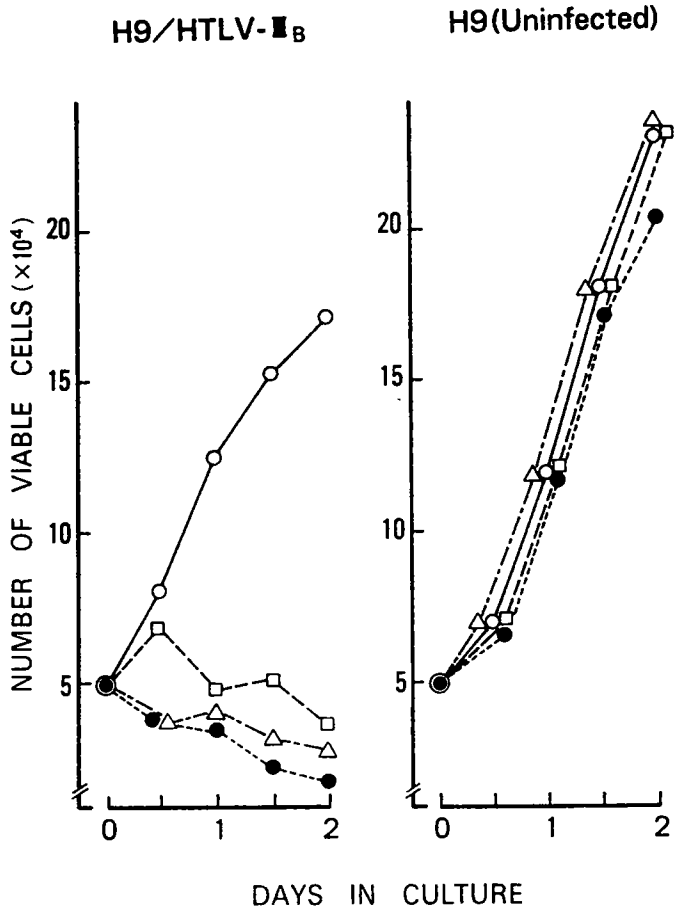

FIG. 1. Selective inhibition of the proliferation of HTLV-IIIB-infected H9 cells (H9/IIIB) by RAC-β. The 0.5β antibody was conjugated with ricin A chain (RAC-β) by SPDP. H9/IIIB cells or uninfected H9 cells were cultured in the presence of RAC-β at concentrations of 4 μg/ml (●), 0.8 μg/ml (△), and 0.16 μg/ml (□). Control culture with no antibody is also shown (○). Total viable cells were counted after 12, 24, 36, and 48 h in culture. When unconjugated 0.5β was added to H9/IIIB cells, no differences in the growth of the cells were observed (not shown). Variabilities in cell number determination is ±10% of the value shown.

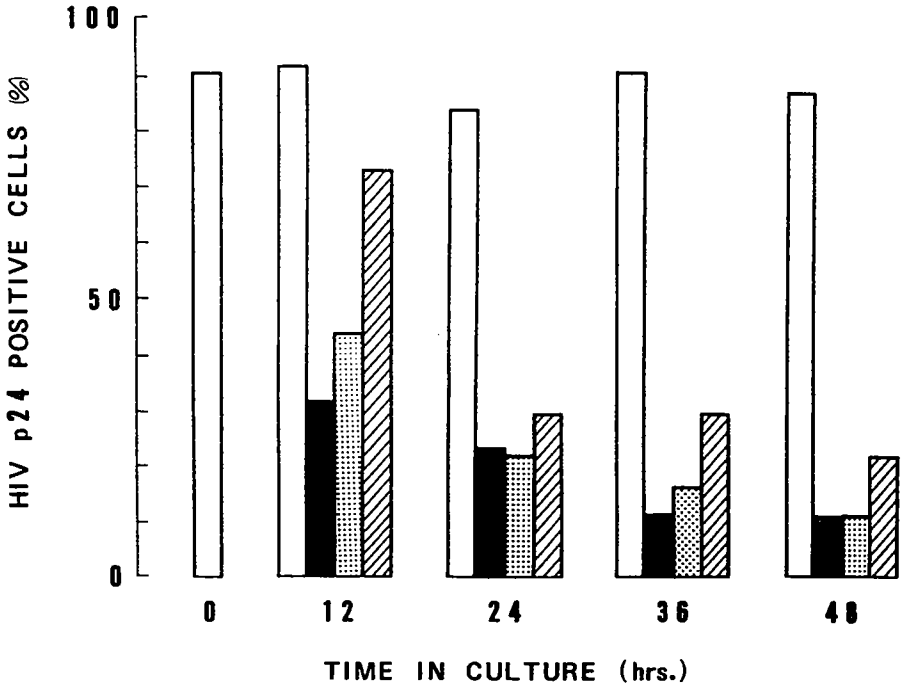

FIG. 2. Selective reduction of the HIV P24-positive cells by RAC-β. H9/IIIB cells were cultured in the absence (□) or presence of RAC-β as described in the legend to Fig. 1 at concentrations of 4 μg/ml (■), 0.8 μg/ml (▩), and 0.16 μg/ml (▨).

Because these toxins are only active when they are internalized by receptor-mediated endocytosis, we investigated surface modulation of gp120 by 0.5β. H9/IIIB cells were cultured for 24 or 48 h, with 10 μg/ml of 0.5β or control antibody, washed, and reacted with 0.5β followed by staining with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody and analyzed by laser flow cytometry. However, no apparent decrease or enhancement in the expression of gp120 on the surface of infected cells was observed despite the presence of 0.5β (data not shown). Taken together, these results, suggest that a part of gp120–0.5β complex may be internalized without apparent up or down modulation of gp120 on the surface of infected cells.

To estimate the feasibility of the 0.5β immunotoxins, we have analyzed humoral immune response to the epitope recognized by 0.5β. A 33 amino acid sequence peptide ABJ709 that contained 0.5β reacting epitope[22] was synthesized and used for the study. Reactivity of diluted sera from HIV asymptomatic carrier (AC) or AIDS-related complex (ARC) were tested by enzyme immunoassay. As shown in Figure 4A, reactivity of the sera with the peptide varies from highly positive to nonreactive in HIV-positive individuals while no reactivity was noted in human T-cell leukemia virus type-I carriers and normal individuals. This result is consistent with the results reported by Palker et al., describing that 21% of seropositive subjects had positive reactivity to a synthetic peptide that partially overlapped with ABJ709.[25] To detect 0.5β epitope specific binding activity, an inhibition assay was employed (Fig. 4B). Of 18 seropositive individuals, 3 had a natural antibody that inhibited the binding of 0.5β to ABJ709. It is worth noting that serum antibodies from these individuals did not show high binding activities to ABJ709 (the actual absorbances were 0.62, 0.28 and 0.17). In view of the finer epitope mapping of 0.5β,[22,26] it is conceivable that other epitopes would be available on ABJ709 to seropositive patients' serum antibodies without interference by 0.5β. These results suggest that at least some seropositive individuals actually produced type-specific antibody and may imply infection of the HTLV-IIIB type viruses.

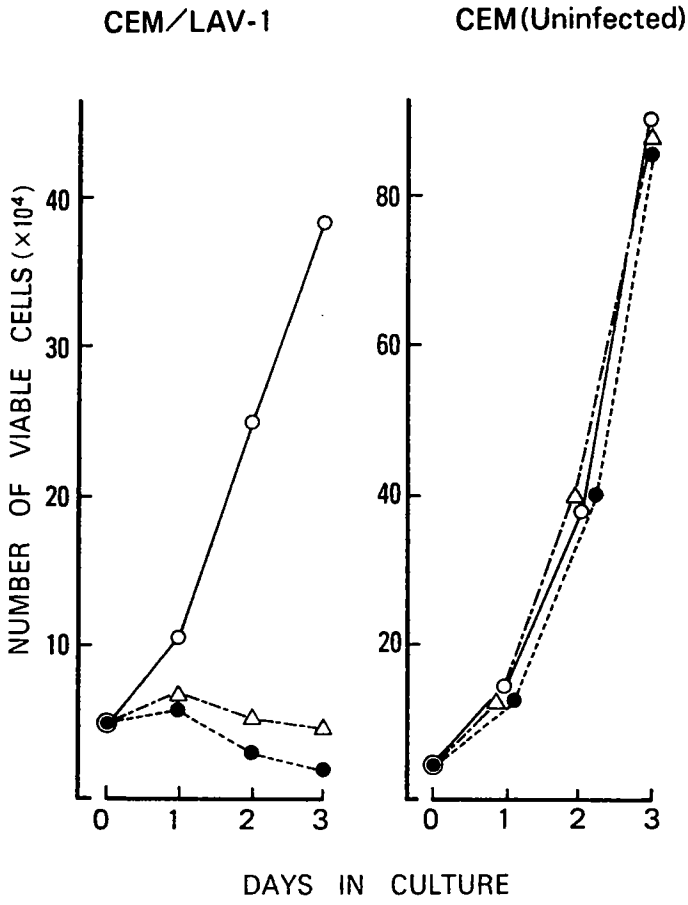

FIG. 3. Selective inhibition of the proliferation of LAV-1-infected CEM subline (CEM/LAV-1) by PE-β. The percent gp120 positivity of CEM/LAV-1 subline was 84% as analyzed by cell sorter using 0.5β (data not shown). CEM/LAV-1 or uninfected CEM cells were cultured in the absence (○) or presence of 1 μg/ml (●), 0.1 μg/ml (△) of PE-β.

Finally, we attempted to detect HIV-bearing cells in freshly isolated peripheral blood mononuclear cells (PBM) by 0.5β. When PBM cells were analyzed by laser flow cytometry, two discrete populations of cells were identified in the cytogram. One of them consists mainly of lymphocytes (region A) and the other of monocytes/macrophages (region B). As summarized in Table 1, when the cells in the B region were examined, binding of 0.5β was detected in 2 of 10 seropositive individuals. No reactivity of 0.5β was observed for the cells in the A region in any of the samples tested. Because the number of cells in the monocyte/macrophage region was about 10% of the whole PBM, the percent positive cells in the PBM population may be 1.8% for Patient 1 and 1.28% for Patient 2.

To investigate whether RAC-β was also effective against patients' infected cells, PBM were cultured with RAC-β and analyzed by flow cytometry using 0.5β. The number of cells in macrophage region (B region) appeared to be decreased, and the reactivity of 0.5β was suppressed after 36 h cultivation with RAC-β (Fig. 5). The percent positive cells in each population in Figure 5 region B was calculated as 3.8% for the background staining, 21% for 0.5β-positive cells, and 4.7% for the 0.5β-positive cells after RAC-β treatment. The percentages of 0.5β-positive cells reduced from 17.2% (21–3.8%) to 0.9% (4.7–3.8%) when cells were exposed to RAC-β. Although it is possible that 0.5β-positive cells may be uninfected cells to which virions attached, RAC-β eliminated gp120-bearing cells in PBM from a patient with ARC in vitro.

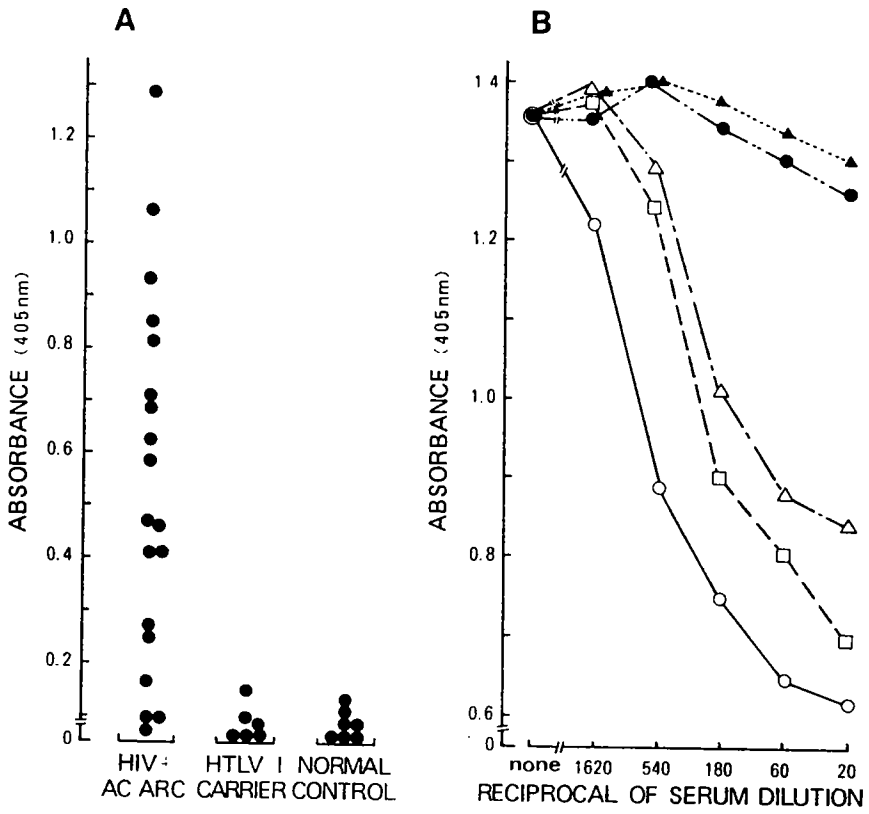

FIG. 4. (A) Reactivity of ARC and AC serum antibodies to a synthetic peptide ABJ709 which contains 0.5β epitope. Binding activity of serum IgG was detected by ELISA. Data were presented by subtraction of background absorbance which was detected as binding to the wells coated with PBS alone from absorbance of antigen-coated wells. Sera from HTLV-I carrier and normal individuals were used as controls. (B) Detection of antibodies that compete in the binding of 0.5β to ABJ709. Serum samples were obtained from two healthy carriers of HIV (○,□), an ARC (△) and two normal donors (●,▲). Data presented as mean value of duplicate determinations.

DISCUSSION

The HIV producing cells (mainly monocyte/macrophages) have been detected in peripheral blood, lymph node, brain, and lung.[6,7,27] Plata et al.[7] reported that 12–58% of the macrophages in bronchoalveolar lavage fluids were positive for HIV. Using scanning/transmission-immunogold techniques, Herrera et al. have detected a high frequency of HIV antigen-bearing cells in PBM cells from HIV-positive patients.[28] The viremic state of HIV infection was detected and found to be associated with the progression of clinical stages by long-term observation of infected individuals.[29] The continuous production of HIV by viral reservoir cells has been considered to play a central role in the progression of HIV-associated diseases.

Here, we demonstrated that the toxin-conjugated anti-gp120 monoclonal antibody selectively killed HIV-infected cells in vitro. The killing effects of the immunotoxins toward gp120-bearing cells in a patient's PBM cells observed in vitro may suggest the possible use of the immunotoxins to eliminate selectively HIV-producing cells in vivo. High titer anti-HIV sera from healthy seropositives were used as an adoptive immunotherapy to treat AIDS patients and showed some effects.[30] Anti-gp120 immunotoxins may have an advantage over other immunotherapy since they destroy HIV-producing cells without participation of effecter cells which may also be susceptible to HIV infection. Anti-gp120 immunotoxin may at least help to reduce viral burden in infected individuals.

TABLE 1. SUMMARY OF CYTOFLUOROGRAPHIC ANALYSIS OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBM) FROM SEROPOSITIVE INDIVIDUALS

PBM donor	*Clinical status*	*T4/T8 Ratio*	*% positive cells*	
			Region A	*Region B*
1	ARC	0.7	1.8	18.1
2	AC	1.0	ND	12.8
3	AC	0.76	< 1	2.8
4	AC	1.09	< 1	< 1
5	ARC	0.6	ND	< 1
6	ARC	0.5	< 1	< 1
7	AC	0.7	< 1	< 1
8	AC	ND	< 1	< 1
9	AC	0.6	< 1	< 1
10	AC	ND	< 1	< 1
Normal PBM (n=2)			< 1	< 1
H9/IIIB			84	
H9			< 1	

[a]PBM were separated on Ficoll-Conray density gradients, stained with 0.5β, and analyzed. The percent positive cells was determined by integration of the fluorescence peak after subtraction of nonspecific background staining.
Abbreviations: ND; not done. ARC; AIDS-related complex. AC; asymptomatic carrier.

Several explanations about the higher frequency of HIV antigen-bearing cells in two patients, compared with that previously reported by Harper et al. are possible.[27] The difference in assay procedure may account for some of the discrepancies. First, indirect immunofluorescence assay (IFA) and in situ hybridization (ISH) used in the previous reports may be less sensitive than flow cytometric analysis. Second, in flow cytometric analysis, the percent positive cells were calculated by integration of the fluorescence peak after subtraction of nonspecific background staining. By contrast, percent positivities in IFA or ISH were calculated from distinct positive and negative cell number. Third, in flow cytometric analysis positivities were calculated within the monocyte/macrophage population by gating in the cytogram, while in IFA and ISH, percent positive cells were calculated in PBM cells as a whole.

Recently, toxins conjugated with soluble CD4 molecule were produced and reported by two groups.[31,32] These CD4 toxins also killed HIV-infected cells in vitro and were shown to be effective against variety of divergent strains of HIV. Because the binding activity of 0.5β is type specific, on the other hand, administration of a toxin-conjugated 0.5β alone may select variant viruses to replicate in infected individuals.[33] It is possible to overcome this problem by producing an immunotoxin that is broadly reactive with a number of HIV isolates or by making a set of type-specific immunotoxins that cover many isolates. Polyclonal and monoclonal antibodies to gp120 that may be able to react with multiple HIV isolates were reported.[34,35] It will be important to see if these group-specific antibodies can also be used to produce effective immunotoxins. The strategy of an immunotherapy against HIV-infected cells by antienvelope immunotoxins may also have an implication in the treatment of other persistent viral infections.

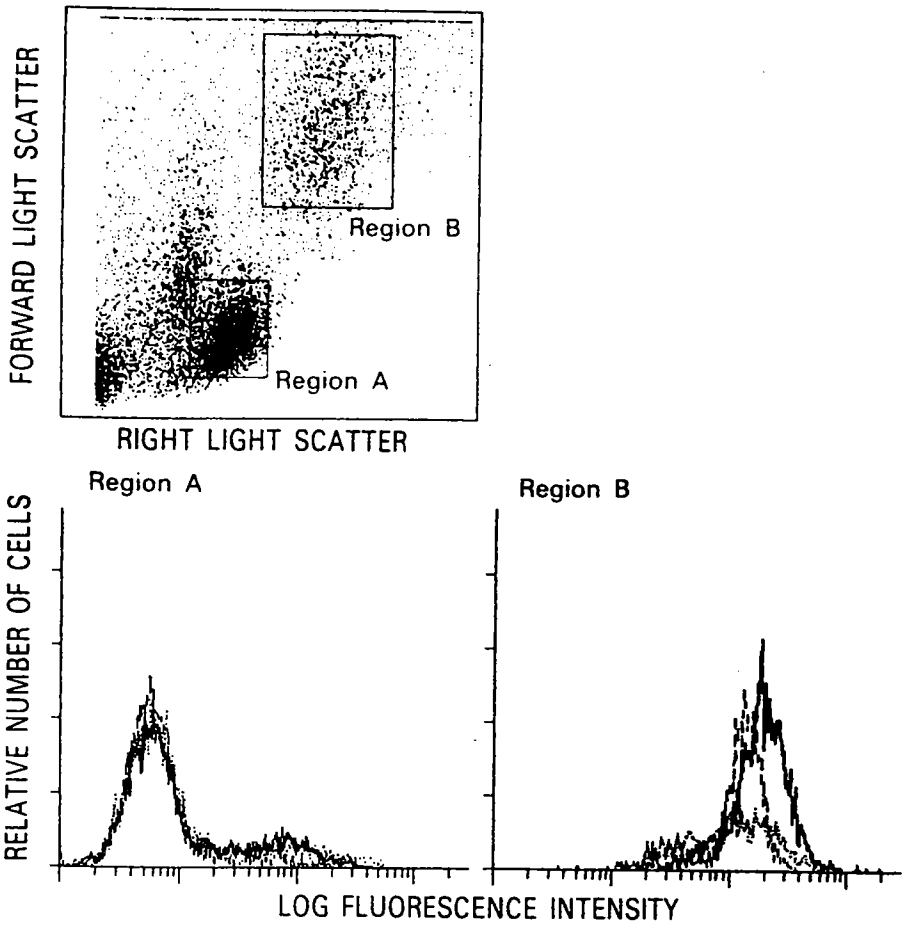

FIG. 5. Elimination of gp120-bearing cells by RAC-β in a PBM cells from an ARC patient in vitro. PBM cells (10^6) from an ARC patient were resuspended in the complete medium containing human IgG and cultured in the presence of the following reagents: sample 1 and 2, MOPC21 (control IgG1); sample 3, 0.5β; sample 4, RAC-β. After 36 h in culture, cells in each sample were collected and reacted with either MOPC21 (sample 1) or 0.5β (samples 2,3,4). The cells were further stained with FITC-conjugated $F(ab')_2$ fragment of anti-mouse IgG antibody and analyzed by laser flow cytometry. The profile of sample 1, stained with MOPC21, is indicated by a broken line. The profile of sample 2, stained with 0.5β, is shown in a solid line. The profile of sample 3, stained with 0.5β, is identical to that of sample 2 (not shown). The profile of sample 4 is shown by a dotted line. Note that RAC-β eliminated the cells in the B region which were positive for 0.5β reactivity. The cytogram (left panel) depicts the population of the cells as mainly consisting of lymphocytes (A region) and monocytes/macrophages (B region).

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EVIDENCE APPENDIX

PART I

EXHIBIT B

Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 3809–3813, April 1994
Medical Sciences

In vitro evolution of a neutralizing human antibody to human immunodeficiency virus type 1 to enhance affinity and broaden strain cross-reactivity

(passive immunization/viral neutralization/phage display/combinatorial libraries/complementarity-determining regions)

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Communicated by Robert M. Chanock, January 4, 1994

ABSTRACT **A method is described that allows for the improvement of antibody affinity. This method, termed complementary-determining region (CDR) walking, does not require structural information on either antibody or antigen. Complementary-determining regions are targeted for random mutagenesis followed by selection for fitness, in this case increased binding affinity, by the phage-display approach. The current study targets a human CD4-binding-site anti-gp120 antibody that is potently and broadly neutralizing. Evolution of affinity of this antibody demonstrates in this case that affinity can be increased while reactivity to variants of human immunodeficiency virus type 1 is broadened. The neutralizing ability of this antibody is improved, as assayed with laboratory and primary clinical isolates of human immunodeficiency virus type 1. The ability to produce human antibodies of exceptional affinity and broad neutralizing ability has implications for the therapeutic and prophylactic application of antibodies for human immunodeficiency virus type 1 infection.**

The ability to clone human antibodies in large numbers from seropositive individuals (1, 2) or to create them *de novo* by using synthetic approaches (3–5) promises increased application of this class of molecules in the service of human health. There are a number of considerations in choosing an antibody-combining site; these are primarily affinity and specificity. Current molecular methods should allow for experimenter-controlled evolution of binding sites to satisfy demands in both areas. The generation of molecules with exceptional affinities should both increase biological potency and decrease the cost of antibodies as therapeutics.

Currently, there is an increased urgency for the development of molecules for the prophylaxis and therapy of human immunodeficiency virus type 1 (HIV-1) infection. Passive immunotherapy has been successfully used against a number of viruses (6) and indeed has been used to protect chimpanzees against HIV-1 infection (7, 8) and to protect cynomolgus monkeys against simian immunodeficiency virus and HIV type 2 infection (9). One of the major problems in using antibodies as anti-HIV-1 reagents is sequence variation in the envelope proteins of the virus. Because the virus requires the binding of the surface glycoprotein gp120 to the CD4 molecule on the target cell for infectivity (10, 11), the CD4-binding site on gp120 has become a popular target for antiviral antibodies (12–16). However, antibodies to this region are not generally particularly potent in terms of virus neutralization. Furthermore, such antibodies tend to be even less potent against primary isolates of virus than the more commonly used laboratory-adapted strains (17). Using combinatorial libraries, we have isolated human anti-CD4-binding site antibodies with quite exceptional neutralizing ability (18). Nevertheless, we wished to improve the likelihood that these antibodies could succeed in prophylactic and therapeutic application.

In the present report we develop a strategy for evolution of antibody affinity. The method is applied to an HIV-1 neutralizing human antibody directed against the CD4-binding site of gp120. For this antibody, which already shows exceptional neutralizing potency, we show the possibility of increasing affinity, potency, and broadening strain reactivity.

MATERIALS AND METHODS

Reagents, Strains, and Vectors. Oligonucleotides were from Operon Technologies (Alameda, CA). *Escherichia coli*, phage, and the phagemid vector pComb3 are as described (19). The recombinant glycoproteins (rgps) 120 IIIB(LAI) and 120 MN were purchased from American Bio-Technologies (Cambridge, MA) and AgMed (Cambridge, MA), respectively. Reagents for surface plasmon resonance experiments were obtained from Pharmacia.

Library Construction and Selection. *Experiment A.* A *Hin*dIII restriction site was introduced preceding the heavy-chain complementarity-determining region (CDR) I by standard methods into clone HIV-4 (18) in the pComb3 vector (19). Clone HIV-4 (Fab b4) and HIV-12 (Fab b12) are identical. This Fab was selected by panning against rgp120 IIIB(LAI). The GenBank accession no. of HIV-4 sequence is L03147. A CDRI library was constructed by PCR of the above construct with primers (*i*) 5′-GAA-GGT-TTC-TTG-TCA-AGC-TTC-TGG-ATA-CAG-ATT-CAG-TNN-SNN-SNN-SNN-SNN-STG-GGT-GCG-CCA-GGC-CCC-C and (*ii*) primer R3B(20), where N is A,C,G, or T, and S is G or C.

The PCR product was gel-purified, digested with *Hin*dIII and *Spe* I, and gel-purified. The product was ligated with *Hin*dIII- and, *Spe* I-digested HIV-4. Subsequent steps were as described (3, 17) to produce phage displaying antibody Fab fragments on their surface. The library, 2×10^7 clones, was affinity-selected by four rounds of panning against gp120 IIIB(LAI) immobilized on Costar 3690 microtiter wells. One well coated with 1 μg of gp120 IIIB was used for each round of selection. After selection, plasmid DNA was prepared, and individual clones were sequenced.

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Abbreviations: CDR, complementarity-determining region; HCDR1 and HCDR3, heavy chain CDR1 or CDR3, respectively; HIV-1, human immunodeficiency virus type 1; PBMC, peripheral blood mononuclear cell; sCD4, soluble CD4; rgp, recombinant glycoprotein.

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Experiment B. Plasmid DNA isolated after experiment A was used as a template for PCR with oligonucleotide primers (*iii*) CCC-TTT-GCC-CCA-GAC-GTC-CAT-ATA-ATA-ATT-GTC-CTG-GGG-AGA-ATC-ATC-MNN-MNN-MNN-MNN-CCC-CAC-TCT-CGC-ACA and (*iv*) FTX-3 (20) to randomize within heavy chain CDR3 (HCDR3) (M = A or C, which gives K = T or G in the complementary strand; NNK doping strategy). The PCR product was gel-purified, digested with *Aat* II and *Xho* I, and gel-purified. This PCR product was ligated with *Aat* II, *Xho* I-digested HIV-4. Subsequent steps were described above to yield a library of 8×10^6 clones. The library was affinity-selected by six rounds of panning against gp120 IIIB. After selection, the gIII fragment was removed, and soluble Fab was produced. The complete amino acid sequences of the variable regions of selected antibodies were deduced by dideoxynucleotide chain-termination sequencing. Fab was purified to homogeneity by affinity chromatography, as described (19).

Surface Plasmon Resonance. The kinetic constants for the binding of Fab to rgp120 IIIB and MN were determined by surface plasmon resonance-based measurements using the BIAcore instrument from Pharmacia. The sensor chip was activated for immobilization with *N*-hydroxysuccinimide and *N*-ethyl-*N'*-(3-diethylaminopropyl)carbodiimide. The proteins, rgp120 IIIB or MN, were coupled to the surface by injection of 50 μl of a 50 μg/ml sample. Excess activated esters were quenched with 15 μl of ethanolamine (1 M and pH 8.5). Typically 4000 resonance units were immobilized. Binding of Fab fragments to immobilized gp120 was studied by injection of Fab in a range of concentrations (0.5–10 μg/ml) at a flow rate of 5 μl/min. The association was monitored as the increase in resonance units per unit time. Dissociation measurements were acquired after the end of the association phase but with a flow rate of 50 μl/min. The binding surface was regenerated with HCl (1 M NaCl and pH 3) and remained active for 20–40 measurements. The association and dissociation rate constants, k_{on} and k_{off}, were determined from a series of measurements as described (20–22). Equilibrium association and dissociation constants were deduced from the rate constants.

Quantitative Infectivity Assay Based on Syncytium Formation. Quantitative neutralization assays with the MN and LA1 (IIIB) strains were done as described (23). Monolayers of CEM-SS target cells were cultured with virus in the presence or absence of Fab, and the number of syncytium-forming units of input virus was determined 3–5 days later. Equivalent amounts of virus were used in the assays to allow direct comparison of Fab concentrations tested. Data represent the average of at least two runs. Assays were repeatable over a virus-surviving fraction range of 1–0.001 within a 2- to 4-fold difference in the concentration of antibody ($P < 0.001$).

Microplaque Neutralization. The quantitative measurement of the reduction of infectivity of primary clinical isolates of HIV-1 was determined with a microplaque assay, as described (24). MT2 cells were used as indicator cells in this assay. The isolation of HIV-1 from frozen peripheral blood lymphocytes obtained from seropositive donors has been described (25). A number of the primary isolates of HIV-1 used in this study have been described (26); these are VL135, VL434, VL069, VL263, and VL596, previously described as isolates 1, 3, 4, [illegible] and 7, respectively.

RESULTS

CDR Walking. In experiment A, the entire heavy chain CDR (HCDR1), as defined by Kabat *et al.* (27), was targeted for mutagenesis using the overlap PCR mutagenesis strategy described (3). NNS- or NNK-type doping strategies were used with no assumptions made as to the most fit residue at each position. After four rounds of selection for binding to rgp120 IIIB, the sequencing of 12 clones indicated a preference for asparagine (N) at position 31, an aromatic residue at position 32, serine (S) or threonine (T) primarily at position 33, branched hydrophobic residues at position 34, and hydrophobic and/or aromatic residues at position 35 (Fig. 1). Experiment B introduced diversity into HCDR3 at positions 96–99 of the clones that survived the four rounds of selection of experiment A. After six rounds of selection for binding rgp120 IIIB, a strong consensus was seen in both mutagenized CDRs (Fig. 1). At the time these selection experiments were done, only rgp120 IIIB was commercially available. Only in case 3B8 was the starting HCDR3 nucleotide and amino acid sequence identical to the parent, indicating some contamination in the secondary library. The net results of this two-step sequential CDR walk were minimal changes from the starting clone. The parental residues at positions 31, 32, 34, and 99 were strongly or absolutely maintained. The hydrophobic parental residue Val-33 was predominantly hydrophilic threonine or serine after selection. Position 96 appears flexible to a variety of substitutions as does position 98. Position 97 shows a preference for the increased steric bulk of the Tyr-97 → Trp mutation.

Affinity Measurement. After selections, four clones were chosen for further study. Clones were chosen that were related to one another by small changes in amino acid sequence and which displayed the most dramatic change in amino acid identity at positions 96 and 98. The kinetics of binding of purified Fab to two types of rgp120 from the highly divergent isolates MN and IIIB were chosen (28). Comparison of the protein rgp120 MN to rgp120 IIIB revealed 88 amino acid changes in the aligned sequences from rgp120 IIIB, as well as 11 deletions and 5 insertions of amino acids. Binding kinetics were studied in real time by using surface plasmon resonance. The kinetic and calculated equilibrium constants are tabulated for the binding of Fabs to both rgp120s (Table 1). The parental clone HIV-4 binds rgp120 IIIB with ≈10-fold better affinity than rgp120 MN, a trend that is maintained for the evolved clones. The highest-affinity Fab, 3B3, is improved 8-fold in affinity to rgp120 IIIB and 6-fold in affinity to rgp120 MN. The increases in affinities of the evolved Fabs binding to rgp120 IIIB and rgp120 MN are well-correlated as shown in Fig. 2. Thus, without selective pressure for binding to rgp120 MN, increase in affinity to

Experiment A	Experiment B		
CDR1	CDR1	CDR3	
31 32 33 34 35	31 32 33 34 35	96 97 98 99	
N F V I H		P Y S W	HIV-4
R Y T V F	N F T L M	Q W N W	3B1
N W S V M	N Y T I M	P W T W	3B2
G Y T L M	N F T V H	E W G W	3B3
N F T L L	N Y T L I	P W N W	3B4
H Y S L M	N F I I M	L W N W	3B6
N W V V H	N F S I M	S W R W	3B7
N F S I M	N Y T I Q	P Y S W	3B8
N F A I H	N F T V H	P W R W	3B9
N F T M V			
N F T L Q			
Y F T M H			
S Y P L H			

FIG. 1. CDR walking for the selection of improved variants of HIV-1. In experiment A, HCDR1, is randomized over positions 31–35. After selection for binding to rgp120 IIIB the sequences listed in experiment A were observed. Experiment B introduces additional diversity into HCDR3 positions 96–99 in clones that were selected in experiment A. After additional selective pressure to bind rgp120 IIIB, the sequences listed under experiment B were observed. The sequences of the parental clone HIV-4 are shown for comparison.

Table 1. Binding and neutralization data for evolved Fab reacting with laboratory isolates of HIV-1

Fab	gp120 type	k_{on}, $M^{-1}s^{-1}$	k_{off}, s^{-1}	K_a, M^{-1}	K_d, M	IC_{50} μg/ml	IC_{50} M
HIV-4	IIIB	7.6×10^4	4.8×10^{-4}	1.6×10^8	6.3×10^{-9}	3.9×10^{-2}	7.7×10^{-10}
HIV-4	MN	3.4×10^4	1.5×10^{-3}	2.3×10^7	4.4×10^{-8}	3.0×10^{-1}	5.9×10^{-9}
3B1	IIIB	8.5×10^4	1.1×10^{-4}	7.7×10^8	1.3×10^{-9}	2.2×10^{-2}	4.4×10^{-10}
3B1	MN	1.4×10^5	1.8×10^{-3}	7.8×10^7	1.3×10^{-8}	9.2×10^{-3}	1.9×10^{-10}
3B3	IIIB	8.4×10^4	6.5×10^{-5}	1.3×10^9	7.7×10^{-10}	4.7×10^{-2}	9.4×10^{-10}
3B3	MN	1.6×10^5	1.2×10^{-3}	1.3×10^8	7.5×10^{-9}	5.5×10^{-3}	1.1×10^{-10}
3B4	IIIB	7.7×10^4	3.6×10^{-4}	2.1×10^8	4.8×10^{-9}	5.0×10^{-2}	9.9×10^{-10}
3B4	MN	8.6×10^4	4.1×10^{-3}	2.1×10^7	4.8×10^{-8}	2.0×10^{-2}	3.9×10^{-10}
3B9	IIIB	4.5×10^4	1.8×10^{-4}	2.5×10^8	5.0×10^{-9}	6.6×10^{-2}	1.3×10^{-9}
3B9	MN	8.1×10^4	1.1×10^{-3}	7.4×10^7	1.4×10^{-8}	7.8×10^{-3}	1.6×10^{-10}

The ability of parental and evolved Fabs to bind rgp120 IIIB and MN was determined by surface plasmon resonance (20–22). The equilibrium association and dissociation constants were calculated from the experimentally determined kinetic constants where $K_a = k_{on}/k_{off}$ and $K_d = k_{off}/k_{on}$. The interpolated IC_{50} values of Fab-neutralizing MN and IIIB viral stocks, as determined with the quantitative infectivity assay based on syncytium formation (23), are given in μg/ml and in molar units.

rgp120 IIIB is accompanied by increased affinity to rgp120 MN. Though a series of single-point mutations is not available in the clones examined to assign changes in affinity directly, comparison of Fab 3B3 with Fab 3B9 and Fab 3B1 with Fab 3B4, which each differ at two positions, suggests change of Pro-96 to glutamine or glutamate as the most productive change. Further examination of acidity changes within the evolved Fabs suggests affinity increases are correlated with decreased pI values of Fabs. The antigens rgp120 IIIB and MN have basic calculated pI values of 9.5 and 9.3, respectively. pI considerations may also contribute to the anomalous behavior of Fab 3B9, which is the only Fab that is increased in pI, as compared with the parent.

Neutralization Studies. Quantitative neutralization assays with the laboratory-adapted strains MN and LAI (IIIB) were done to determine potency of the Fabs (23). As shown in Fig. 3*A*, the evolved Fabs are clearly improved in their abilities to neutralize infectivity of the MN viral stock. The binding affinity of Fabs to rgp120 MN is well-correlated with ability to neutralize the MN stock (Fig. 4). The highest-affinity Fab, 3B3, is improved 54-fold with respect to neutralization of the MN isolate in this assay (Table 1). A different MN viral stock was used in these studies than had been used in the initial characterization of Fab HIV-4 (18). This result is reflected in a difference in activity, as compared with this previous report. Studies with the LAI (IIIB) viral stock show a clustering of Fabs with similar potencies (Fig. 3*B*). With this viral stock a range of reactivity of only 3-fold is noted with the most potent Fab, 3B1, showing a modest 2-fold increase in potency. With both viral stocks, Fabs demonstrate exceptional potency in the 10^{-10} M range (Table 1). To further

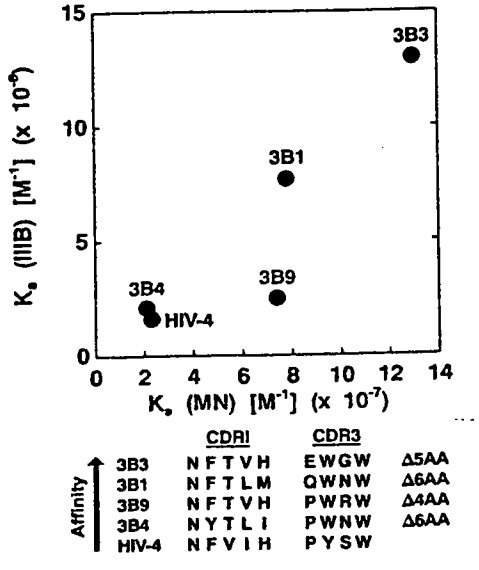

FIG. 2. The affinity increases of evolving Fabs for binding the divergent envelope proteins rgp120 IIIB and rgp120 MN are well-correlated. Affinities were determined by using the surface plasmon resonance technique (20–22). The sequences of evolved clones are ranked as compared with the parent, and changes in the amino acid (AA) sequence from the parent are shown as ΔAA. See also Table 1.

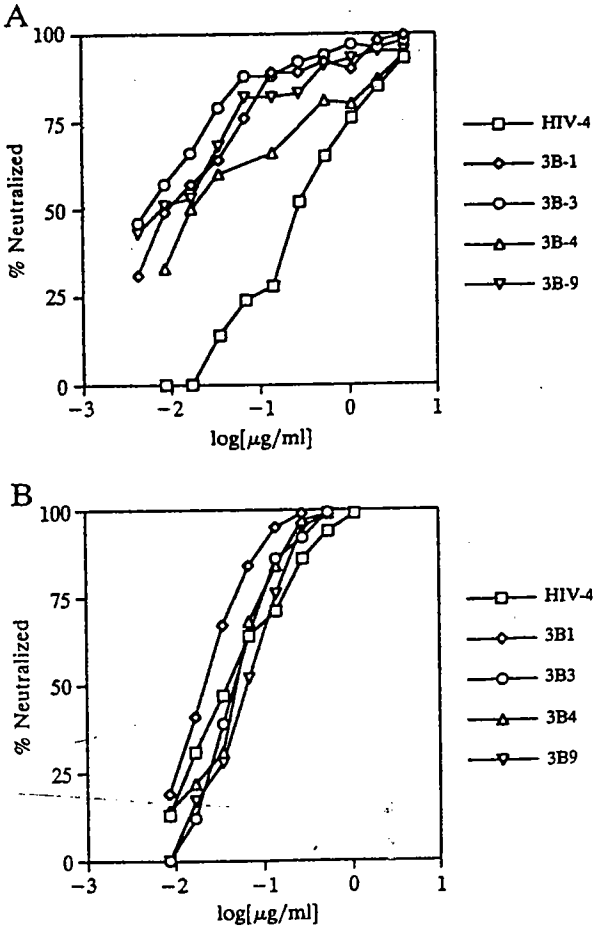

FIG. 3. Evolved Fabs are potent in neutralization assays with laboratory isolates of HIV-1. (*A*) Parental and improved Fabs are compared in a quantitative infectivity assay based on syncytium formation (23) with MN viral stock. (*B*) Comparison with LAI (IIIB) viral stock. Results indicate the average of at least two assays.

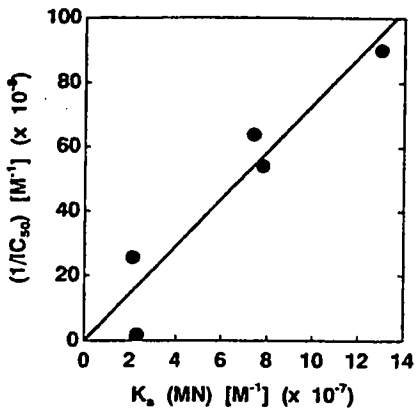

FIG. 4. Affinity increases are well correlated with increased potency in the quantitative infectivity assay with the MN viral stock. IC_{50} values for the reduction of infectivity were interpolated from Fig. 3*A*. The equilibrium association constant for binding rgp120 MN was determined as in Table 1.

delineate functional changes that accompany increased affinity, neutralization studies with primary clinical isolates of HIV-1 were done. Primary clinical isolates of HIV-1 were grown in peripheral blood mononuclear cells (PBMCs) (25). As controls in this microplaque assay (24), IIIB and MN stocks, as well as isolate VL069, were propagated in H9 cells. Additionally, assays with all stocks were done with a pooled plasma from 13 HIV-1-seropositive individuals. In all cases MT2 cells were used as indicator cells. The results are shown in Table 2. In these assays, the highest-affinity Fab, 3B3, is now able to neutralize an additional four isolates, as compared with the parent Fab, HIV-4. Fifty-percent neutralization of isolates VL135 and VL530 by Fab 3B3 at 38.9 and 29.5 μg/ml, respectively, is significant because the parent, Fab HIV-4, showed insignificant levels of neutralization, $\approx$10%, at 50 μg/ml. Neutralization of these isolates with pooled positive plasma shows that these isolates are relatively resistant to neutralization, as compared with the laboratory isolates grown in H9 cells. Neutralization of MN and IIIB stocks by antibody 3B3 is improved $\approx$5-fold in this assay. A host-cell effect is noted with isolate VL069. Propagation of

Table 2. Neutralization of primary clinical isolates of HIV-1 with natural and evolved Fab

Virus	Host cell	Amount for 50% neutralization, μg/ml		Titer + PHP
		HIV-4	3B3	
VL135	PBMC	>50	38.9	1:33
VL263	PBMC	17.0	6.6	<1:10
VL596	PBMC	33.1	17.0	1:10
VL069	PBMC	>50	>50	<1:10
VL434	PBMC	>50	10.5	1:10
VL114	PBMC	>50	5.2	<1:10
VL172	PBMC	>50	>50	1:10
VL530	PBMC	>50	29.5	<1:10
VL750	PBMC	>50	>50	1:10
IIIB	H9	0.36	0.068	1:767
MN	H9	0.18	0.044	1:24,000
VL069	H9	3.6	3.5	1:1,200

The potency of the HIV-4 parental clone and Fab 3B3 to neutralize primary clinical isolates was measured in a microplaque assay (24). Virus was either propagated in PBMC or H9 cells. The neutralizing ability of pooled human plasma from 13 HIV-1-seropositive patients (+PHP) is shown for comparison as the titer of the serum dilution. Furthermore, the laboratory-adapted stocks IIIB and MN were also tested in this variant assay for comparison. A host-cell effect is shown for isolate VL069 grown in H9 cells (29).

this isolate in H9 cells results in a sensitization to neutralization. This effect has been noted previously and is discussed in detail elsewhere (29). In these assays the intrinsic error of the interpolated titers averages ±30%.

DISCUSSION

The present study shows the feasibility of improving antibody affinity and function where specific structural information on both antibody and antigen is not available and the antibody already possesses high affinity. The current approach termed "CDR walking" is a variant of our synthetic-antibody approach for the generation of additional specificities *in vitro* (3–5). Practically, there is one important difference. CDR walking involves a limited introduction of diversity into the CDR regions of a defined antibody, as contrasted with the synthetic approach for the generation of new specificities where structural diversity is stressed over library completeness. Diversity in the present case is limited to 6 or fewer amino acid residues with an NNK- or NNS-doping strategy so as to ensure near-complete representation of all possible amino acid combinations. Selection from the library with the phage-display technique then allows for the refinement of the contact between antigen and antibody, which may result from unpredictable sequence changes in the region of interest. Repeated introduction of diversity into CDRs followed by stringent selections should allow for the refinement of human antibodies to levels of affinity far beyond those generated by the immune response. Two strategies are evident for the application of this approach, either sequential or parallel optimization of CDRs. Parallel optimizations makes the assumption that the optimized loops will exhibit additivity in free energy changes when the individually optimized loops are combined (30, 31). In many cases, additivity will likely be observed. Sequential optimization takes into account that additivity may not always be observed and that optimal binding may result from the interdependence of loops. Such interdependence could result from coordinated structural changes on binding antigen and is supported by recent evidence that suggests induced-fit mechanisms may best describe antibody–antigen recognition (32, 33). The two-step sequential walk reported here demonstrates the potential of this strategy. Both sequential and parallel approaches are being examined at present. In this initial study HCDR1 and HCDR3 were chosen for optimization. These CDRs were targeted because rearrangements of these CDRs have been observed on binding of another antibody to antigen (33). Residues 96–99 of HCDR3 were targeted because chain-shuffling experiments indicated this region is a hotspot during the natural maturation of this antibody (34). After the randomization and selection protocol that sampled mutations of 9 amino acid positions, higher-affinity Fabs resulted that had mostly modest changes from the parent (Fig. 1). The most radical change involves the Pro-96 → Glu mutation found in the highest-affinity clone 3B3, which is 8-fold improved in binding rgp120 IIIB. Interestingly, this mutation is also observed in the *in vivo* response, as revealed by chain shuffling. The *in vivo* antibodies that share this mutation are, however, of lower affinity than HIV-4 and have accumulated many additional somatic mutations throughout their sequence (34).

The key issue in producing antibodies to HIV-1 for therapeutic or prophylactic purposes is that they should be highly potent (of high affinity and neutralizing ability) and be cross-reactive with a wide range of isolates. These are usually two opposing characteristics. We have chosen HIV-4, as it recognizes a conformational epitope on gp120 that overlaps the CD4-binding site of gp120 and is broadly and potently active (19). If HIV-4 is truly recognizing the conserved features (shape) of the CD4-binding region, it should be

possible to increase its affinity to gp120 for many or all viral strains, as to date all HIV-1 isolates use CD4 as their primary receptor. This phenomenon is, indeed, observed as shown in Fig. 2. Binding has been increased to MN and IIIB, two highly divergent isolates (28). Selective pressure could have been applied to favor cross-reactivity by selecting with a mixture of divergent gp120s; however, this did not prove to be necessary in the present investigation. The present strategy was dictated because only rgp120 IIIB was commercially available when the selection experiments were done. Potency as judged by quantitative neutralization assays with MN and LAI (IIIB) stocks is improved as well (Fig. 3). With the MN isolate affinity is well-correlated with neutralizing ability (Fig. 4). Neutralization of MN and LAI stocks with soluble CD4 (sCD4) revealed IC_{50} values of 0.6 nM and 0.8 nM, respectively (35). As shown in Table 1, the parental clone and Fab 3B1 have IC_{50} values of 0.8 nM and 0.4 nM, respectively, for the LAI stock. For MN, the parental clone and Fab 3B3 have IC_{50} values of 6 nM and 0.1 nM, respectively. The ability of these evolved monovalent Fabs to neutralize with potencies equivalent to sCD4 is distinctive. The lack of correlation of rgp120 IIIB affinity with neutralizing ability may reflect the sensitivity of the assay conditions in this range. In a recent multicenter study of human and mouse anti-HIV-1 antibodies, no bivalent antibody has demonstrated such potency (36).

Can a single CD4-site antibody fulfill the promises once made by sCD4 as a therapeutic agent? Primary clinical isolates often require 1000-fold more sCD4 for neutralization than laboratory isolates (37–39). This fact may be the primary contributor to the failure of sCD4. As shown in Table 2, the highest-affinity Fab also demonstrates improved ability to neutralize primary clinical isolates. Four isolates not neutralized by the parent are now neutralized by Fab 3B3. For isolate VL114 a titration with bivalent CD4 IgG, predicted to have high activity, yielded an IC_{50} of 10 μg/ml, as compared with 5.2 μg/ml for Fab 3B3. As most of the CDR residues have yet to be optimized, it should be possible to further evolve this Fab to affinities 100 or 1000 times those reported here. These preliminary results suggest that broadly reactive antibodies of exceptional affinity can be prepared. Such antibodies will likely be of use at least to prevent vertical transmission of virus and in cases of accidental exposure. Ideally a mixture of such antibodies directed against several epitopes would be used. It remains to be demonstrated whether antibodies alone can be effective in cases where lymph nodes and thymus are seeded with the virus; however, they will likely be valuable components in combination therapies (40) and perhaps as key targeting agents in future therapies.

We thank Richard A. Lerner for support and encouragement of this project; Terri Jones and Kim Green for protein purification; and Pamela Morden, Mary Kate Morris, and Leo Ocequerq for technical support in primary isolate neutralization assays. C.F.B. is a Scholar of the American Foundation for AIDS Research and the recipient of a Scholar award from the Cancer Research Institute. Support for this work was provided by National Institutes of Health Grant AI33292 and Johnson & Johnson.

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EVIDENCE APPENDIX

PART I

EXHIBIT C

US005458878A

United States Patent [19]

Pastan et al.

[11] Patent Number: 5,458,878

[45] Date of Patent: Oct. 17, 1995

[54] **P. EXOTOXIN FUSIO PROTEINS HAVE COOHG220101AL ALTERATIONS WHICH INCREASE CYTOTOXICITY**

[75] Inventors: **Ira Pastan**, Potomac; **Vijay K. Chaudhary**, Rockville; **David Fitzgerald**, Silver Spring, all of Md.

[73] Assignee: **The Government of the United States of America as represented by the Secretary of the Department of Health and Human Services**, Rockville, Md.

[21] Appl. No.: **522,563**

[22] Filed: **May 14, 1990**

Related U.S. Application Data

[63] Continuation-in-part of Ser. No. 459,635, Jan. 2, 1990, abandoned.

[51] **Int. Cl.**[6] **A61K 39/104**; C07K 3/00; C07K 15/28; C12P 21/08

[52] **U.S. Cl.** **424/260.1**; 424/279.1; 435/69.7; 530/387.3; 530/391.7

[58] **Field of Search** 424/85.91, 260.1, 424/279.1; 435/69.7; 530/387.3, 391.7

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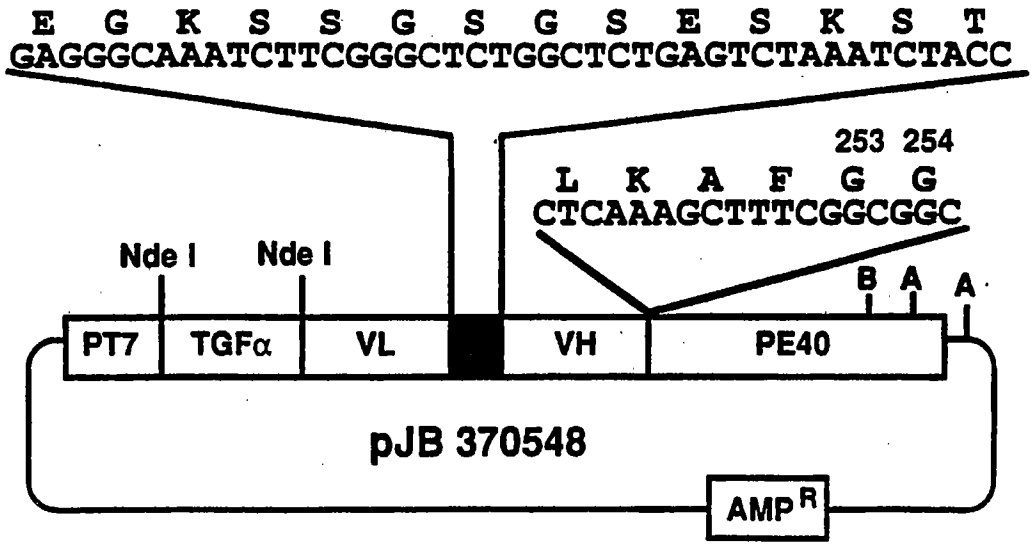
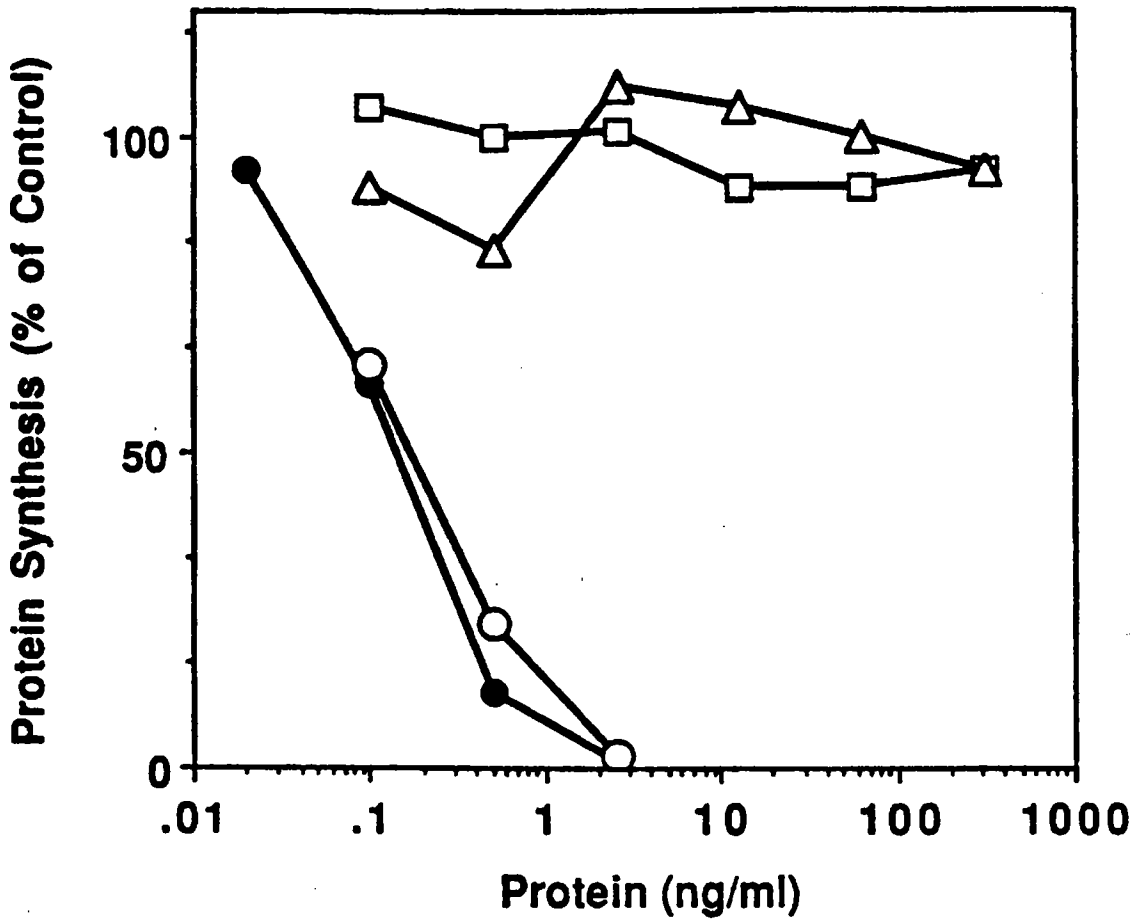
Primary Examiner—David L. Lacey
Assistant Examiner—T. Michael Nisbet
Attorney, Agent, or Firm—Townsend and Townsend and Crew

[57] **ABSTRACT**

A target-specific, cytotoxic, recombinant Pseudomonas exotoxin is described. Such toxins are made by inserting specific recognition molecules at specific cloning sites in at least domain III near the carboxyl terminus of the PE molecule. Various modifications of the carboxyl terminus of the PE molecule to increase cytotoxicity are set forth. Multifunctional, recombinant, cytotoxic fusion proteins containing at least two different recognition molecules are provided for killing cells expressing receptors to which the recognition molecules bind with specificity. Methods for producing novel recombinant PE molecules with specific properties are described.

29 Claims, 4 Drawing Sheets

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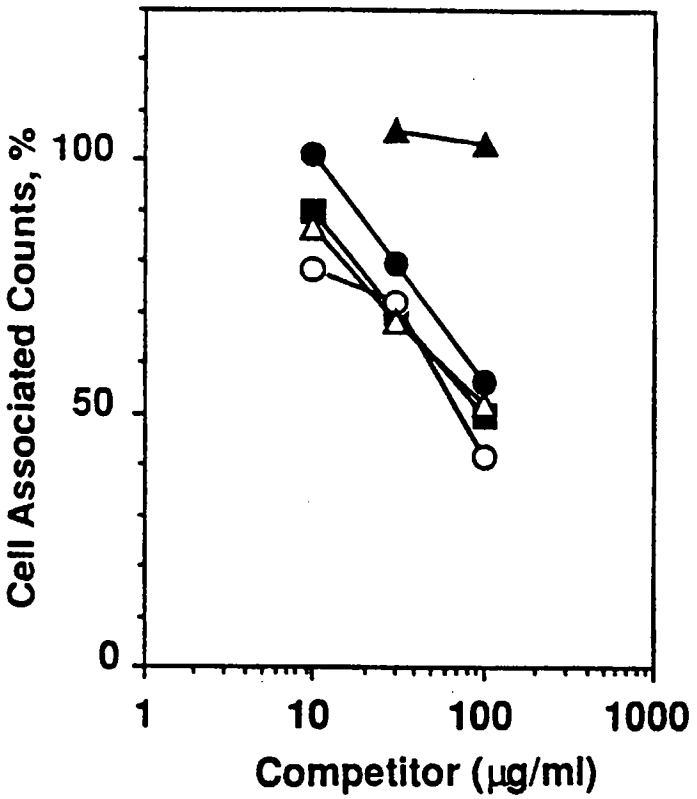
.1
1
10
100
1000
Protein (ng/ml)

FIG. 1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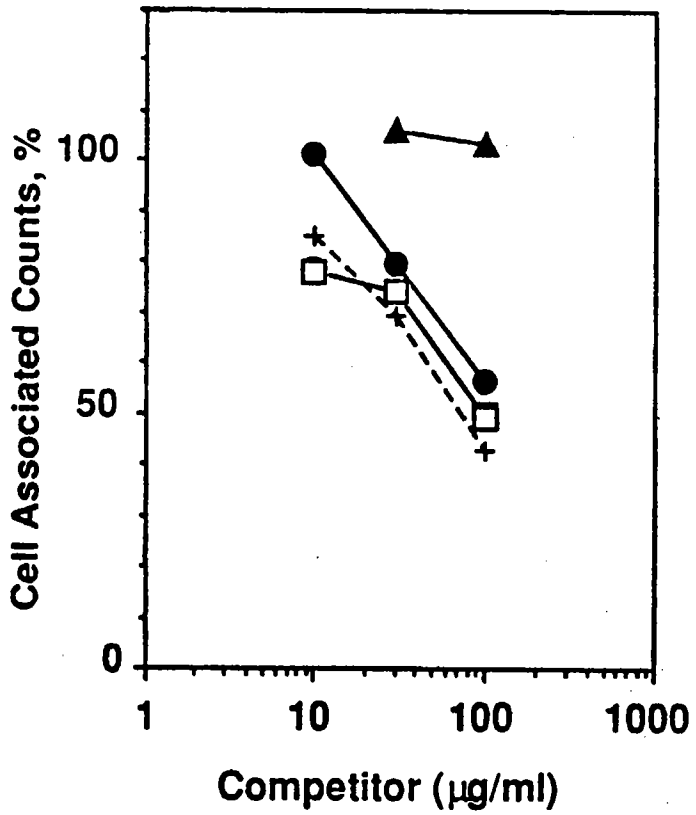
1
10
100
1000
Competitor (μg/ml)

FIG. 2A


Cell Associated Counts, %
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1
10
100
1000
Competitor (μg/ml)

FIG. 2B

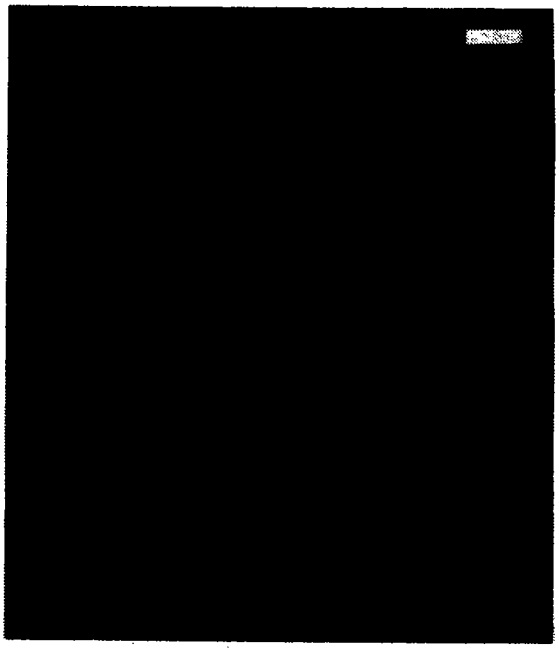


FIG. 3A

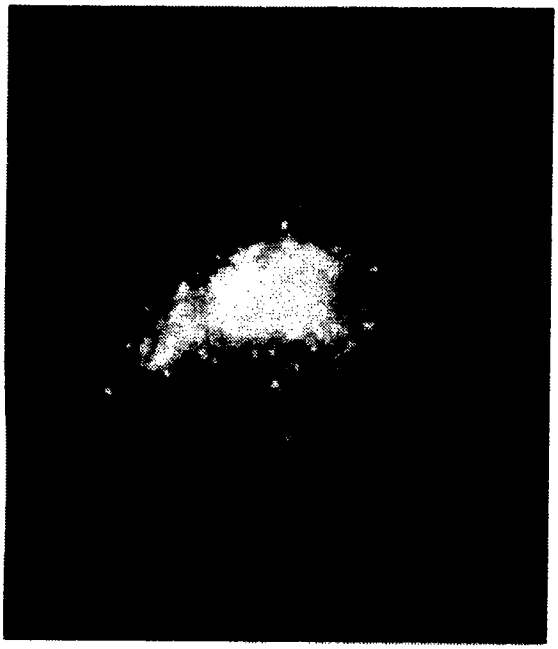


FIG. 3B

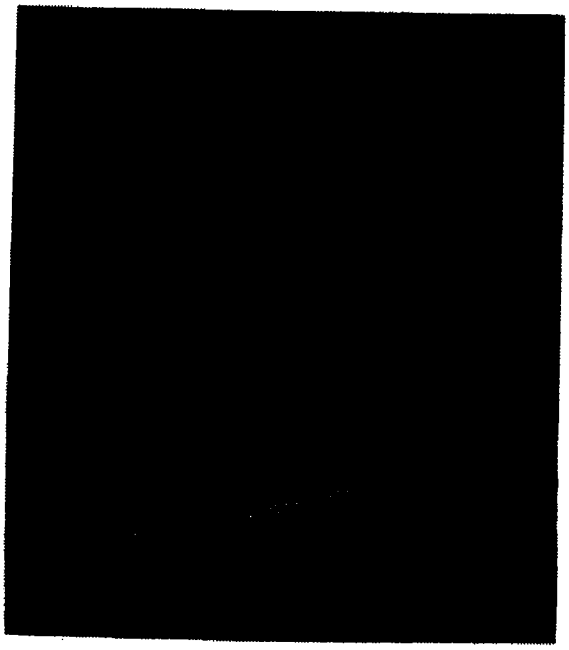
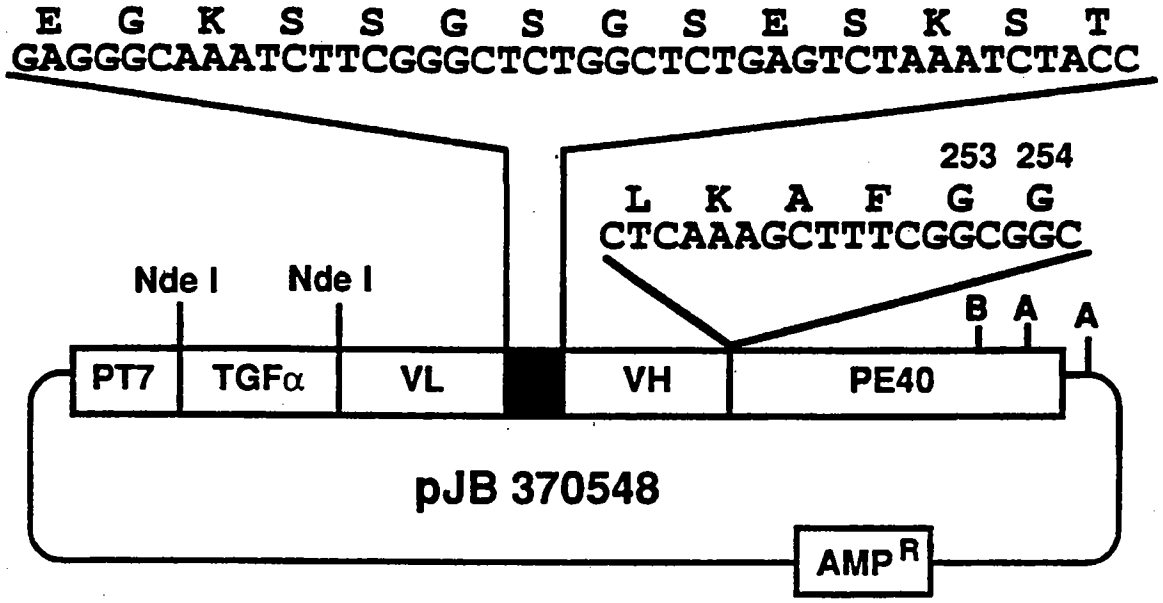


FIG. 3C

FIG. 3D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Nde I
B A A
PT7
TGFα
VL
VH
PE40
pJB 370548
AMP R

FIG. 4

P. EXOTOXIN FUSIO PROTEINS HAVE COOHG220101AL ALTERATIONS WHICH INCREASE CYTOTOXICITY

This a continuation in part of the application Ser. No. 07/459,635, filed Jan. 2, 1990, now abandoned.

The present invention is related generally to the making of improved recombinant immunotoxins. More particularly, the present invention is related to the construction of a recombinant Pseudomonas exotoxin (rPE) with specific cloning sites for the insertion of recognition molecules at least at the carboxyl end of the PE to achieve target-directed cytotoxicity and for the construction of recombinant multifunctional chimetic cytotoxic proteins.

BACKGROUND OF THE INVENTION

The mechanism by which protein toxins kill cells is quite complex. Many toxins bind to receptors on the surface of mammalian cells, are internalized by endocytosis, translocate to the cytosol and there exert an enzymatic activity that kills the target cell. Accordingly, these toxins have separate domains for cell binding, translocation and an enzymatic activity that lnactivates an essential cellular function. Pseudomonas exotoxin-A (PE) is a single polypeptide chain of 613 amino acids. X-ray crystallographic studies and mutational analysis of the PE molecule have shown that PE consists of three domains: an amino terminal cell receptor binding domain (Domain I); a middle translocation domain (Domain II); and a carboxyl terminal activity domain (Domain III). Domain III catalyzes the ADP ribosylation and inactivation of elongation Factor 2(EF-2) which inhibits protein synthesis and leads to cell death. Mutational analysis of Domain I has revealed that lysine^{57} plays a major role in receptor binding. Similarly glutamic acid^{553}, Tyrosine^{481} and histidine^{426} have been shown to be important for ADP-ribosylation activity. Recently mutational analysis of domain II has shown that certain portions of this domain are absolutely required for the cytotoxicity of PE.

While constructing various chimetic toxins in which growth factors were fused to a form of PE (PE40) which was devoid of domain I, it was observed that the recombinant fusion proteins, made by attaching TGFα, interleukin-2 or interleukin-4 at the carboxyl end of PE40 had poor cytotoxic activity. Hence, an examination of the role of the carboxyl terminus of the PE molecule (domain III) was undertaken.

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to determine the role of the carboxyl terminus of the PE molecule in its cytotoxic action.

It is another object of the present invention to identify specific regions at the carboxyl terminus of the PE molecule for the insertion of recognition molecules for selective killing of target cells.

It is a further object of the present invention to provide an improved, target-specific, cytotoxic recombinant PE molecule, wherein the improvement comprises the insertion of target-specific recognition molecule at least in domain III at the carboxyl terminus of the PE molecule.

A still further object of the present invention is to modify the carboxyl end of the PE to increase the potency of the chimeric toxin.

It is yet another object of the present invention to make cytotoxic PE with two recognition molecules (target ligands)

wherein either the same recognition molecule is placed at two different termini, such as the amino terminus and near the carboxy terminus. for enhanced cellular binding, or two different recognition elements are inserted, one each at two different regions of the PE molecule, to enable more effective binding of the resulting PE molecule to the cell surface having two or more different entities. such as antigens, receptors and the like to which said recognition elements could bind.

It is an additional object of the present invention to provide a recombinant PE having repeat carboxyl end sequences for enhanced cytocidal activity.

An additional object of the present invention is to provide a multifunctional, recombinant cytotoxic chimetic protein for simultaneously killing cells expressing different types of receptors.

Various other objects and advantages will become evident from the following detailed description of the invention.

ABBREVIATIONS

Various abbreviations. symbols, terminologies and the like used herein are now set forth.

PE-40 means a PE molecule of about 40,000 Mr.(Hwang et al, **1987**. Cell 48:129–136) having a deletion of domain I.

TGFa-PE40 means a chimetic protein wherein TGFa is the targeting or recognition molecule linked to PE-40. When the targeting agent is a different entity such as CD4 and the like, the chimetic protein is accordingly designated CD4-PE40 and the like.

When a numbering system is used, such as PE-Gly609, it means that the amino acid at position 609 in the sequence of the native PE has been replaced by glyclne. The same convention is used throughout the specification. The symbol means the deletion of amino acids following the symbol.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

FIG. **1** demonstrates cytotoxicity of PE and PE mutants on Swiss cells. Various dilutions of PE proteins were made in PBS containing 0.2% human serum albumin and added to 1×10^5 Swiss 3T3 cells in 24-well plates. Sixteen hours later the cells were pulse labeled with 3H-leucine and TCA predictable cell associated radioactivity was determined as a measure of protein synthesis. The results are expressed as percent of control where no toxin was added. ●—● PE; ○—○ PEΔ613; □—□ PEΔ612,613; and Δ—Δ PEΔ611–613. All the assays were done in duplicate and repeated twice.

FIGS. **2A–2B** show the results of competition for the cellular uptake of recombinant PE. Swiss 3T3 mouse cells were incubated with 400 ng ^{3}H-PE (specific activity 3.5×10^5 DPM/μg) and increasing concentrations of purified mutant proteins for one hour at 37° C. Cell monolayers were washed and cell-associated radio-activity was determined. ●—●PE; ▲—▲PEglu57; Δ—Δ PEΔ612,613; ○—○PEΔ613; ■—■ PEgly^{276}; □—□ PEΔ609–613; ●—●PEΔ609–613●598–613.

FIGS. **3A—3A** show immunofluorescence detection of binding and internalization of Pseudomonas exotoxin and its recombinant variants in Swiss 3T3 cells. Swiss 3T3 cells

were incubated at 37° C. for 30 minutes in the absence of toxin FIG. 3(A) or in the presence of 10 μg/ml of native Pseudomonas exotoxin (PE) FIG. 3(B), recombinant PE gly^{57} FIG. 3(C) or recombinant PEΔ612,613 FIG. 3(D). Following this incubation, the cells were fixed in formaldehyde and further incubated in the continuous presence of saponin. The cells were incubated with mouse monoclonal anti PE (M40-1) (10 μg/ml), followed by affinity-purified rhodamine-labeled goat anti-mouse IgG (25 μg/ml). (Mags–×400; bar=10 μm).

FIG. 4 schematically shows the construction of a multivalent recombinant cytotoxic fusion protein using for illustration herein TGRα and anti-Tat genes. The expression plasmid pJB370548 contains a fusion gene encoding the first 50 amino acid of TGFα, the variable domain of anti-Tac light chain (VL, first 106 amino acids of anti-Tac light chain), a 14 amino acid linker, the variable anti-Tac heavy chain (VH, first 116 amino acids of heavy chain), and amino acids 253–613 of PE. AmpR, β-lactamase gene: B, BamH1; A, AvaI. Amino acids are shown as single letter codes. The gene is under the control of the bacteriophage T7 promoter linked to a Shine-Dalgarno sequence and an initiation codon.

DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the present invention are achieved by a cytotoxic recombinant Pseudomonas exotoxin (rPE) having a recognition molecule inserted at least in domain III at the carboxyl terminus of the PE for selective killing of target cells recognized by said recognition molecule without substantial cytotoxicity to other cells not recognized by said recognition molecule, and by a rPE with modified "cytotoxic sequence" with increased cell killing activity. A multifunctional fusion protein having versatility, flexibility and efficacy for killing eel is expressing different types of receptors is provided.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference. Unless mentioned otherwise, the techniques employed or contemplated herein are standard methodologies well known to one of ordinary skill in the art. The materials, methods and examples are illustrative only and not limiting.

The term "recognition molecule" as defined herein means those molecules or ligands which recognize only target cells desired to be killed. Examples of such recognition molecules are antibodies or portions thereof that can recognize the target cells, growth factors, lymphokines, cytokines, antigens, hormones and the like or combination thereof, which specifically bind to molecules on the surface of the target cells.

The term "cytotoxic sequence" as used herein means those variety of amino acid sequences at or near the carboxyl end of the PE, the presence of which is a prerequisite for the cytocidal activity of the toxin and the repeat sequences of which may determine the level of cytotoxicity. The examples of such sequences are KDEL, REDLK and the like as will become apparent from the various embodiments of the sequences discussed herein below.

The term "multivalent" cytotoxic recombinant fusion protein as used herein means that the recombinant fusion protein has at least two similar or different recognition molecules for receptors expressed on the target cells, to which either the first, the second or both recognition molecules bind with particular specificity. Of course, the multivalent fusion protein may be made with any of a variety of cytotoxic sequences described herein.

MATERIALS AND METHODS

Materials

Unless mentioned otherwise, the materials and reagents used herein were obtained from commercial sources. Polymerization Chain Reaction (PCR) kit, Gene Amp Kit, was obtained from Perkin Elmer-Cetus, Norwalk, Conn.

Mutants and Plasmid Constructions

Mutants were created by oligonucleotide directed mutagenesis using plasmid pVC45f+T as described by Jinno et al, 1988, *J. Biol. Chem.* 263, 13203–13207 and Jinno et al, 1989, *J. Biol. Chem.* 264, 15953–15959 or using polymerase chain reaction (PCR) as described below. pVC45f+T carries a PE gene under a T7 promoter and also contains a T7 transcriptional terminator and a f1 phage origin. The PE gene also contains an OmpA signal sequence which is cleaved upon secretion of PE into the perlplasm leaving a 3 amino acid (ala asn leu) extension at the amino terminus (Chaudhary et al, 1988, *Proc. Natl. Acad. Sci. USA* 85, 2989–2948). For PCR mutagenesis, two oligonucleotides and a 1.0 Kb SalI-EcoRI fragment of pVC45f+T were employed. One oligonucleotide was the same as nucleotides 2216–2236 of the PE gene (Gray et al, 1984, *Proc. Natl. Acad. Sci. USA* 81, 2645–2649). Other oligonucleotides were complementary to the 3' end of the coding sequence PE gene, contained desired mutations and created an EcoRI site after the stop codon. Other unique restriction sites were also created without changing amino acids to identify the mutants. A 30 cycle PCR was performed with denaturation at 94° C. for 2 minutes, annealing at 55 C. for 1 min and polymerization at 72 C. for 3 min with 10 seconds extension per cycle using a gene amplification thermal cycler (Perkin Elmer Cetus). After the PCR, amplified fragment was cut with EcoRI and BamHI. it was purified using low melting point agarose. PCR fragments were ligated with a 4.5 Kb dephosphorylated EcoRI-BamHI fragment of pVC45f+T. Mutants were identified by unique restriction sites which were created during mutagenesis and finally confirmed by sequencing by Sanger's dideoxy-chain termination procedure using Sequenase (US Biochemical Corp.).

pVC4915f+T

This plasmid contains two mutations: Codon 608, CCG and 609, CGC were changed to CCC and GGG, respectively. This mutation results in glycine at 609 in place of arginine and creates a SmaI site between codons 608 and 609. This plasmid was used to clone various carboxyl terminal fragments of PE. pVC4975f+T: A 1 Kb BamHI-PstI fragment of pVC8 (Wozniak et al, 1988, *Proc. Natl. Acad. Sol. USA* 85, 8880–8884) was restricted with NarI, treated with T4 DNA polymerase to make blunt ends followed by EcoRI, and a 286 bp fragment was ligated to a 4.9 Kb dephosphorylated SmaI-EcoRI fragment of pVC4915f+T. pVC4985f+T: A 1 Kb BamHI-PstI fragment of pVC8 was restricted with HinfI, treated with T4 DNA polymerase followed by EcoRI and a 237 bp fragment was ligated to the 4.9 Kb SmaI-EcoRI fragment of pVC45f+T. pVC4995f+T: A synthetic oligonucleotide duplex VK192/193 (not shown), containing

codons 598–613 of PE with a stop codon and an EcoRI compatible 3' end, was ligated to the 4.9 Kb SmaI-EcoRI fragment of pVC4915f+T. pVC4715f+T: This plasmid was created by PCR mutagenesis and contains restriction sites StuI, NdeI, SmaI, EcoRV and EcoRI within the 3' end of the PE gene and encodes amino acids RPHMPGDILK in place of PREDLK at 608 to 613. These unique sites were later used to make

DNA segments encoding insertions and to attach various carboxyl terminal portions of PE.

pVC47195f+T

This was created by ligating oligonucleotide duplex VK191/192 to a 4.9 Kb EcoRV-EcoRI fragment of pVC4715f+T. The carboxyl terminus of this PE mutant contains amino acids RPHMPGDPDYASQPGKPPREDLK in place of amino acids 608–613 (PREDLK) of PE.

Plasmids for the insertion of receptor binding domains in the carboxyl end of PE

Plasmid pVC4715f+T consists of DNA sequences encoding PE from amino acids 1 to 607 followed by a polylinker that contains StuI, NdeI, SmaI, EcoRV and AflII sites as well as encodes amino acids RPHMPGDILK. These sequence are under T7 promoter control and also contain Shine-Dalgarno region and signal sequence from OmpA of *E. coli*. Plasmid pVC4715/4E f+T is similar to pVC 4715 f+T, but also contains mutations in the receptor binding domain of PE (Domain I). These mutations are Lys^{57}→Glu, $His^{246,249}$→Glu and Arg^{247}→Glu.

Plasmid pVC 47195/f+T is similar to pVC4715 f+T but contains a polylinker with StuI, NdeI, SmaI sites encoding amino acids RPHMPGI followed by the last 16 codons of PE that encode PDYASQPGKPPREDLK. Plasmid pVC47195/4Ef+T contains insertions of a cDNA encoding transforming growth factor α in the NdeI site of plasmids pVC 4715f+T and 4715/4Ef+T respectively. Plasmids pVC 47395f+T and pVC 47195/4Ef+T are derived from 47195f+T and pVC 47195/4Ef+T by inserting TGFα sequences into the NdeI site. Plasmid pVC 47355/4Ef+T was derived from pVC47395/4Ef+T by deleting 6 amino acids, inserting TGFα and following it with 10 amino acids of the PE carboxyl end.

A deposit of pVC49415f+t and pVC47355/4Ef+t has been made at the ATCC, Rockville, Md., on Dec. 28, 1989 under accession numbers 68198 and 68199, respectively. The deposit shall be viably maintained, replacing if it becomes non-viable during the life of the patent, for a period of 30 years from the date of the deposit, for for 5 years from the last date of request for a sample of the deposit, whichever is longer, and upon issuance of the patent made available to the public without restriction in accordance with the provisions of the law. The Commissioner of the Patents and Trademarks, upon request, shall have access to the deposit.

Protein Expression and Purification

Cultures of *E. coli* strain BL21 (λDE3) containing various plasmids were grown to OD650 of 0.6–0.8 and induced with 1 mM isopropyl-thiogalactoside for 90 minutes at 37° C. Periplasmic fractions were prepared as described by Chaudhary et al, supra. By virtue of having an OmpA signal sequence, more than 90% of each of the expressed toxin proteins were secreted into the periplasm. These proteins have a residual ala asn leu sequence at the amino end left behind after the processing of the OmpA signal sequence. Periplasmic fractions were assayed for ADP ribosylation activity and cytotoxicity. Later PE mutants were purified using a MonoQ anion exchange column (HR5/5) attached to a Pharmacia FPLC system. PE and mutant proteins eluted at NaCl concentrations of 0.22–0.26M. Upon SDS-PAGE the toxins were more than 90% pure. Protein concentration was measured by the Bradford assay Reagent (BioRad.Richmond. Calif.) using bovine serum albumin as a standard.

ADP Ribosylation and Cytotoxicity Assays

ADP ribosylation activity was assayed after the activation of PE and mutant proteins with 4M urea and 50 mM DTT unless otherwise stated (Collier et al, 1971, *J. Biol. Chem.* 246, 1496–1503). Cytotoxicity of PE mutants was determined by adding various dilutions of periplasmic proteins or purified proteins to 1×10^5 Swiss 3T3 cells in 24-well plates as described by Jinno et al, (1988) supra, and Jinno et al, (1989) supra. ADP-ribosylation and cytotoxic activities of recombinant PE and native PE (from Swiss Serum and Vaccine Institute, Berne, Switzerland) were indistinguishable.

Toxin Binding and Internalization

The ability of various mutant PE proteins to compete the binding of ^{3}H-labelled PE on Swiss cells as well as internalization of various mutant PE derivatives as studied by immunofluorescence has been described by Jinno et al, (1989) supra.

Preparation of Target-Specific Immunotoxins

PE expression vectors with the cloning sites in the carboxyl end of PE to produce selective cytotoxic molecules are illustrated here with TGFa, a recognition molecule which recognizes only EGF receptor bearing cells. These cloning sites were used to insert TGFα near the carboxyl end of PE which produced a very active molecule that killed EGF receptor bearing cells when the insertion was followed by the last 10 amino acids of PE (Table A). The details of the procedure are now described.

RESULTS

The role of sequences at the carboxyl end of PE was determined by making a series of carboxyl terminal deletion mutants that removed 1,2,3,7,8,11,14 and 24 amino acids. Removal of two or more amino acids eliminated cytotoxicity without affecting ADP ribosylation activity (Table 1, FIG. 1). In fact, even eleven amino acids (603–613) could be removed without any loss of ADP ribosylation activity. However, removal of 14 amino acids resulted in a protein with low, but measurable, ADP ribosylation activity (600–613) and removal of 24 amino acids (590–613) produced an enzymatically inactive protein. These results indicate that particular sequences at the carboxyl end of PE have a role in toxin action that are not required for ADP ribosylation activity.

The role of the carboxyl terminal sequences in toxin action was defined by creating a series of internal deletions and substitutions (Table 2). These mutations began at amino acid 602 so that ADP ribosylating activity would not be affected and extended to position 611. It was found that several small deletions encompassing amino acids 601–604 and 606–608 did not reduce cytotoxicity. Furthermore, two substitutions which altered amino acids 603–608 as well as two other substitutions within amino acids 606–608 of PE did not reduce cytotoxicity. Therefore, the sequence of amino acids in positions 602–608 did not appear to be important for cytotoxicity. However, deletions which removed arginine at 609 (pVC 49215 and pVC 49255) greatly reduced the cytotoxic action of PE. These results, together with the experiments in Table 1 showing that deletion of amino acids 612 and 613 abolish cytotoxicity, focused our attention on amino acids 609–613 which are situated at the carboxyl terminus of PE.

The role of arginine 609 was studied by either deleting it

or replacing it with several different amino acids. Replacement of arginine at 609 with another basic amino acid, lysine, retained the cytotoxic activity of PE (Table 3). However, deleting arginine at 609 (pVC 49115) or replacing it with glycine, glutamic acid or leucine reduced cytotoxicity about 6–10-fold. Thus, a basic amino acid appears to be important at position 609.

To study the sequence specificity of the last five amino acids of PE, several other mutant molecules were then constructed. In two of these, the order of the acidic amino acids at positions 610 and 611 was reversed and lysine 613 deleted (Table 4, pVC 49415 and pVC 49425). These molecules were fully active whether or not position 609 was a lysine or an arginine. A molecule was also created with a leucine at position 609 and an arginine at 612 (pVC 49435) that was inactive.

Although deletion of the terminal amino acid lysine at 613 did not affect cytotoxicity, it was suspected that other mutations in this position might affect cytotoxicity in a negative manner because of the low activity of various chimetic toxins in which the ligand was placed in peptide linkage at the carboxyl terminus of PE. Therefore, lysine^{613} was converted to glutamine, asparagine or aspartate. All these mutations produced a less cytotoxic molecule (Table 5). Addition of 6 or 11 amino acids to the carboxyl terminus of PE also produced a less cytotoxic molecule (data not shown). However, replacement of lys^{613} with the basic amino acid, arginine, did not decrease cytotoxicity. Thus, positions 609 and 613 both require a basic amino acid for full cytotoxic activity. There are two other lysine residues at the carboxyl end of PE; these are situated at positions 590 and 606. both of these lysines could be converted to the uncharged amino acid glutamine without a decrease in cytotoxicity, indicating that a positively charged amino acid was not required at position 590 or 606 (Table 5).

Having shown the importance of particular amino acids at the carboxyl terminus of PE, it was determined that the five carboxyl terminal amino acids could be separated from the ADP ribosylation domain to regenerate an active toxin. As shown in Table 6, a fully active cytotoxic molecule could be generated from PE 609–613 (which is not cytotoxic) by the addition of amino acids 551–613, 567–613 or 598–613 of an intact PE to the carboxyl terminus of PE 609–613. Thus, the distance between the ADP ribosylation domain which ends around amino acid 600 and the essential amino acids at positions 609–613 was not critical and could be substantially increased without a decrease in cytotoxicity. Also shown in Table 6 is a PE molecule with the carboxyl terminus of RPHMPGDILK in place of PREDLK. This molecule, in which arg^{609} and asp^{611} were altered, was not cytotoxic. But attaching the last 16 amino acids of an intact PE molecule to give a carboxyl terminus of RPHMPGDPDYASQPGKP-PREDLK restored cytotoxicity to this molecule.

Furthermore, constructs were made in which a cDNA TGF was inserted at the carboxyl end of PE with an inactive carboxyl terminus (Table A pVC 47815/4Ef+T) and an active carboxy]terminus (Table A, pVC 47355f+T and pVC 47395f+T). The constructs with good carboxyl termini were more than 50 times as cytotoxic to cells with EGF receptors (TGFα binds to the EGF receptor) as the ones with the bad carboxyl ends. This clearly indicates that for the highest cytotoxic activity, a suitable carboxyl end is an essential requirement.

Altogether the data presented herein demonstrate that the cytotoxic activity of a PE molecule that is inactive due to a deletion or modification within the carboxyl end can be restored by attaching an intact carboxyl end. Hence, it is now possible to create active chimetic molecules by inserting a binding ligand such as TGFα at 608 within the carboxyl end of PE thus retaining the last five amino acids as REDLK.

Although it has been previously demonstrated that domain I of PE is the region responsible for cell binding, it was important to show that the mutations at the carboxyl end of PE that decreased cytotoxicity did not also somehow decrease cell binding. To test this, the ability of various mutant forms of PE to compete for the uptake of [^{3}H]-PE was evaluated. As shown in FIG. 2, several PE mutants that had decreased cytotoxicity due to mutations at the carboxyl terminus of PE were just as able to compete for the uptake of [^{3}H]-PE as authentic wild type of PE. In this competition assay, PE40 which has a deletion of domain I and PEglu^{57} were inactive as previously described (Jinno et al, supra).

These uptake results were confirmed using a fluorescence assay that measured the internalization of PE and various mutant PE molecules (FIG. 3). In this assay, cells are incubated with various toxins for 30 minutes to allow binding and internalization into endocytic vesicles. Molecules with a point mutation in domain I (PEglu^{57}) or PE40 were not internalized. In contrast, all the other PE molecules. whether or not they contained mutations at the carboxyl end of domain III, were found to have bound and internalized into endocytic vesicles and other elements in the trans-Golgi system in the perinuclear area of the cells (FIG. 3, Panel B and D). These results clearly show that decreased cytotoxicity of carboxyl terminal mutants is not due to decreased receptor binding or cellular uptake of PE molecules.

In summary, the results presented herein clearly show that mutations at the carboxyl end of PE and particularly in the last five amino acids of PE result in a molecule with full ADP ribosylation activity, but greatly reduced cytotoxicity. The data show that the amino acid sequence at the carboxyl end of PE is Arg, Glu, Asp, Leu, Lys (REDLK, Table 2). The arginine at 609 can be replaced by lysine but non basic amino acids cannot be tolerated (Table 3). Lysine at 613 is not essential and can be deleted without loss of cytotoxic activity (Table 1), but it cannot be replaced with a non-basic amino acid (Table 5). Thus, having either ArgGluAspLeu or LysGluAspLeuLys at the carboxyl terminus produced a fully cytotoxic molecule (Table 4). A search of the literature for similar sequences that were present in other molecules and performed a specific biological function revealed that the sequence which retains newly formed proteins within the endoplasmic reticulum is LysAspGluLeu (Munro et al, 1987, *Cell* 48, 899–907). Therefore, several other mutant molecules were constructed, one of which contained the exact sequence previously described as being responsible for the retention of the protein in the lumen of the endoplasmic reticulum (Table 4). It was found that a molecule ending with LysAspGluLeu (KDEL) was fully cytotoxic. Also a molecule ending in ArgAspGluLeu (RDEL) but not LeuAspGluArg (LDER) was fully active. These findings indicate that the successful entry of PE into the cytosol from an endocytic compartment requires interaction with the similar cellular component that helps retain proteins made by the cells within the endoplasmic reticulum. These findings also suggest that the sequence at the carboxyl end of PE acts as some type of recognition sequence to assist translocation of PE from an endocytic compartment into the cytosol. Other sequences that perform the same function would likewise increase the activity.

Of further significance was the finding that because the cell targeting ligands can be inserted at two cloning regions in the PE molecule (at the amino terminus as previously

described or near the carboxyl end as described herein), the same or different targeting ligands can be inserted at these two regions thereby increasing either cell binding, cytotoxicity or both. Different targeting molecules at each of the two cloning regions would enable the chimetic toxin to bind to two different types of receptors present on the same cell. This is important because some antigens on target cells do not internalize well and are, therefore, poor targets for immunotoxins. But, if the chimeric toxin or immunotoxin also binds to another antigen that is well internalized, specific cell killing is increased greatly.

It was further discovered during the modificational study of the carboxyl end of the PE that if the REDLK (single letter amino acid code) sequence is replaced with KDEL, the resulting molecule is about two fold more active. Even more spectacular was the finding that a molecule with three repeats of KDEL in place of REDLK. was three times as active (Table B) indicating that by adding KDEL or equivalent repeat sequences, chimetic toxins with enhanced cytotoxicity can be produced.

In short, the present invention for the first time shows that:

1. An appropriate carboxyl end sequence is absolutely required for cytotoxicity of the PE;
2. Deletion of as few as two amino acids from the carboxyl end of PE yields a molecule that contains full ADP ribosylation and receptor binding activity, but is nontoxic to target cells (Table 1);
3. Mutational analyses indicate that PE should possess a positively charged amino acid at 609, negatively charged amino acids at 610 and 611 and a leucine at 612;
4. Lysine at 613 can be deleted but cannot be substituted with several other amino acid residues;
5. Addition of random amino acid residues at the carboxyl end of PE produce relatively inactive molecules (data not shown).
6. Addition of at least 10 carboxyl end amino acids of PE to the PE molecules that are not cytotoxic due to mutations in the carboxyl end, restores full cytotoxic activity (Table 4);
7. Different targeting ligands at different ends (amino and carboxyl) provide the flexibility of producing better binding and cytocidal PE molecules; and
8. Repeat "cytotoxic sequences" multiply the cytotoxicity in appropriate cases.

Of course, other target-specific immunotoxins are made similar to the method described herein supra by using appropriate recognition molecules, toxins and cytotoxic sequences including such modified recombinants as TGFα-PE40, CD4-PE40 and the like (See Table C). An example of a bifunctional toxin, cytotoxic for cells expressing two different receptors is now provided in accordance with the present invention to illustrate such constructions. It may be noted in this respect that conventional immunotoxins and chimetic toxins usually made in bacteria are ordinarily directed to only one receptor or antigen on target cells. The successful construction of an active chimetic toxin molecule containing more than one target recognizing entities, opens the possibility of producing chimetic molecules of greater versatility, flexibility and efficacy. FIG. 4 shows the schematic construction of pJB370548 to produce a multipurpose chimetic protein containing two recognition molecules, TGFa and anti-Tac (Fv) for binding to cells expressing either EGF, IL2 or both. Table D shows a comparison of the cytotoxic activity of this novel multifunctional recombinant fusion protein compared to monofunctional entities tested against appropriate cells easily suggested to one of ordinary skill in the art.

A deposit of pJB 370548 has been made at the ATCC. Rockville, Md., on Apr. 30, 1990 under accession number.

The deposit shall be viably maintained, replacing if it becomes non-viable during the life of the patent, for a period of 30 years from the date of the deposit, for for 5 years from the last date of request for a sample of the deposit, whichever is longer, and upon issuance of the patent made available to the public without restriction in accordance with the provisions of the law. The Commissioner of the Patents and Trademarks, upon request, shall have access to the deposit.

Of course, a method of preparing a cytotoxic, recombinant PE in accordance with the present invention, comprises the steps of utilizing the plasmid described herein without alteration or modifying said plasmid to contain a desired DNA sequence and then functionally insetting said plasmid in an expression vector so that a desired cytotoxic recombinant PE is produced and then recovering the desired PE in a substantially pure form. It is noted that the procedures for modifying, expressing and obtaining the desired PE from the plasmids are quite standard in the art and easily suggested to one of ordinary skill, given the teachings contained herein.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various changes and modifications in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

TABLE 1

Deletion analysis of the carboxyl terminus of PE

Mutants			
amino acids present	amino acids deleted	Cytotoxicity	ADP ribosylation activity
1–589	590–613	<0.1	0
1–599	600–613	<0.1	20
1–602	603–613	<0.1	100
1–605	606–613	<0.1	100
1–606	607–613	<0.1	100
1–610	611–613	<0.1	100
1–611	612,613	<0.1	100
1–612	613	100	100
1–613		100	

Legend to Table 1. Mutant PE proteins were expressed in *E. coli* using T7 promoter based vector (Studier and Moffatt, 1986) and purified from the periplasm. All proteins contain a 3 amino acid (ala asn leu) extention at the amino terminus remaining after the processing of the OmpA signal sequence. These amino acids were not considered when assigning residues-numbers to the above mutant proteins. Cytotoxicity was determined by assaying inhibition of protein synthesis on Swiss 3T3 mouse cells. All results are expressed as percent of the activity obtained with recombinant full length PE molecules. All the assays were done in duplicate and at least 2 separate clones were tested.

TABLE 2

Internal deletions and substitutions within the carboxyl terminus of PE.

	Location of the amino acids in PE												
Plasmid PVC	660	660	660	660	660	660	660	660	660	661	661	661	Cytotoxicity
	1	2	3	4	5	6	7	8	9	0	1	2	3	
45	A	S	Q	P	G	K	P	P	R	E	D	L	K	100
49215	A											L	K	<0.1
49235	A				G	K	P	P	R	E	D	L	K	100
49245	A	S	Q	P	G				R	E	D	L	K	100
49255	A	S	Q	P	G					E	D	L	K	0.3
4955	A	S	Q	P	G	P	K	P	R	E	D	L	K	100
4935	A	S	G	S	H	L	A	A	R	E	D	L	K	100
4955	A	S	E	G	K	S	S	G	R	E	D	L	K	100
49315	A	S	Q	P	G	M	M	M	R	E	D	L	K	100

Mutant PE proteins were expressed in *E. coli* and purified from the periplasm. ADP ribosylation activities of all the mutants were indistinguishable from the full length PE.
Amino acids within the carboxyl end of PE (601–613) are shown as single letter code. The substitutions have been underlined.

TABLE 3

Mutations at position 609 of PE

Plasmid (pVC)	Mutant Proteins	Cytotoxicity (% of PE)
49115	PEΔ609	12
49125	$PELys^{609}$	100
4915	$PEgly^{609}$	10
49135	$PEglu^{609}$	16
49155	$PEleu^{609}$	15

Mutant PE proteins were expressed in *E. coli* and purified form the periplasm. Substitutions are shown as replacement amino acids (also see Tables 1 and 2).

TABLE 4

Sequence specificity of last 5 amino acids of PE

Plasmids	Location of the amino acid in PE					Cytotoxicity
(PVC)	609	610	611	612	613	(% of PE)
45	R	E	D	L	K	100
49125	K	E	D	L	K	100
4215	R	E	D	L		100
49415	K	D	E	L		100
49425	R	D	E	L		100
49435	L	D	E	R		<0.03

For details see legend to Tables 1 and 2.

TABLE 5

Mutations of the lysines residues 590, 606 and 613 in the carboxyl terminal domain of PE

Mutants	Cytotoxicity (% of PE)	ADP-ribosylation (% of PE)
$PEΔ^{613}$	100	100
$PEarg^{613}$	100	100
$PEgln^{613}$	1	100
$PEglu^{613}$	1	100
$PEasn^{613}$	1	100
$PEgln^{606}$	100	100
$PEgln^{590}$	100	100
$PEgln^{590,606,613}$	1	100
$PEgln^{590,606}arg^{613}$	100	100

Analyses were performed as described in Tables 1 and 2.

TABLE 6

Addition of various portions of PE carboxyl terminus to PEΔ609–613

Plasmid (pVC)	Mutant Proteins	Cytotoxicity (% of PE)	ADP-ribosylation activity (% of PE)
4905	PEΔ609–613	<0.1	100
4975	PEΔ609–613+551–613	100	100
4985	PPΔ609–613+567–613	100	100
4995	PEΔ609–613+598–613	100	100
4715	PEΔ609–613 RPHMPGLILK	<0.1	100
47195	PEΔ608–613 RPHMPGD+598–613	50	100

Legend to Table 6. A plasmid pVC4915 with a SmaI site between codons 608 and 609 of PE was created and various portions of the carboxyl terminus were attached after codon 608. pVC4995 was constructed using synthetic oligonucleotides. The last 16 amino acids (598–613) of PE consists of PDYASQPGKPPREDLK (also see Table 1 and 2). Δ means the deletion of amino acids following the symbol.

TABLE A

Cytotoxic activity on A431 cells of various carboxy terminal insertions of TGFα into PE.

Plasmid	Protein[a]	ID_{50}(ng/ml)[b]
pVC47315/ 4E(f+)T	PE 1–607 RPHMA (TGFα) AHMPGDILK	>25
pVC47395/ 4E(f+)T	PE 1–607 RPHMA (TGFα) AHMPGIPDYASOPGKPPREDLK	0.5
pVC47355/ 4E(f+)T	PE 1–607 RPHMA (TGFα) AHMPGKPPREDLK	0.5

[a]Fusion proteins were partially purified from periplasm. SDS-PAGE indicated that the fusion proteins were 20–30% pure. Residues normally present in PE are underlined.
[b]ID_{50} is the concentration of fusion protein (estimated as total protein concentration) that is required to inhibit protein synthesis by 50 percent as compared to control where no toxin was added. Protein synthesis was measured by 3H-leucine incorporation.

TABLE B

Cytotoxic activity on Swiss 3T3 cells of various PE derivatives

Plasmid	Protein[a]	ID_{50} (ng/ml)
pVC 45f+T	PE 1–608 REDLK	1.6
pVC 49415f+T	PE 1–608 KDEL	0.76
pSS 49445f+T	PE 1–608 KDELKDELKDEL	0.55

[a]PE proteins were purified on Mono Q column and were approximately 90% pure.
[b]Same as Table A.

TABLE C

I. ACTIVITY OF TFGα-PE40 AND KDEL DERIVATIVES (ID_{50}) ON CELLS WITH EFG RECEPTORS.

	A431 ng/ml	KB ng/ml	OVCAR 3 ng/ml	HUT 102 ng/ml
TGFa-PE40	.35 .44	.96	5.4	>312
TGFa-PE40 KDEL*	.048 .034	.37	.84	>312
TGF4-PE40 $(KDEL)_3$**	.076 .022	.12	1.1	>312

*TGFa-PE40 (253–609 KDEL)
**TGFa-PE40 (253–609 KDEL KDEL KDEL)
This TABLE shows that replacing the last 5 amino acids of TGFα-PE40 with KDEL or $(KDEL)_3$ increases its activity 3 to 10-fold.

II. CYTOTOXICITY OF CD4-PE40 DERIVATIVES ON ENV-5 CELLS THAT EXPRESS gp120 OF HUMAN IMMUNODEFICIENCY VIRUS.

PROTEIN	ID_{50} (ng/ml)
CD4-PE40+REDLK	2.5
CD4-PE40+KDEL	0.5
CD4-PE40+$(KDEL)_3$	0.65

Increased cytotoxicity of CD4-PE40 on target cells expressing HIV gp120 produced by replacing the last 5 amino acids of CD4-PE40 with KDEL or $(KDEL)_3$. ENV-5 cells express gp120.

TABLE D

Comparison of cytotoxic activity of bifunctional and monofunctional recombinant fusion proteins.

	ID_{50}[a] (ng/ml)	
	HUT102	A431
TGFα-anti-Tac(Fv)-PE40	7.8	12.0
Anti-Tac(Fv)-PE40	2.3	~500
TGFα-PE40	>500	0.5

[a]ID_{50} is the concentration of the fusion protein that gave 50% inhibition of protein synthesis.

What is claimed is:

1. A fusion protein comprising a recombinant Pseudomonas exotoxin (PE) molecule, a first recognition moiety for binding a target cell, and a carboxyl terminal sequence of 4 to 16 residues which permits translocation of said fusion protein into the target cell cytosol, the first recognition moiety being inserted in domain III of PE after residue 600 and before residue 613.

2. The fusion protein of claim **1**, wherein the carboxyl terminal sequence comprises, in a direction from the amino terminus to the carboxyl terminus, the following amino acid residues:

$$R^1—R^2—R^3—R^4—(R^5)_n$$

wherein,

R^1 is a positively charged amino acid residue;

R^2 is a negatively charged amino acid residue;

R^3 is a negatively charged amino acid residue;

R^4 is L; and

R^5 is a positively charged amino acid residue;

and wherein n is zero or 1.

3. The fusion protein of claim **2**, wherein R^1 is selected from the group consisting of R and K.

4. The fusion protein of claim **2**, wherein R^2 is selected from the group consisting of E and D.

5. The fusion protein of claim **2**, wherein R^3 is selected from the group consisting of D and E.

6. The fusion protein of claim **2**, wherein n is 1 and R^5 is selected from the group consisting of K and R.

7. The fusion protein of claim **2**, wherein the carboxyl terminal sequence is selected from the group consisting of REDLK, KEDLK, REDLR, REDL, and KDEL.

8. The fusion protein of claim **2**, wherein the carboxyl terminal sequence is KDELKDELKDEL.

9. The fusion protein of claim **2**, wherein the first recognition molecule is an antibody or a portion of an antibody which recognizes the target cell.

10. The fusion protein of claim **2**, wherein the first recognition molecule is selected from the group consisting of a growth factor, lymphokine, cytokine, and a hormone.

11. The fusion protein of claim **2**, wherein the first recognition molecule is TGFα or CD4.

12. The fusion protein of claim **2**, wherein the first recognition molecule is inserted after residue 607 of the PE molecule.

13. The fusion protein of claim **2**, wherein a second recognition molecule is inserted in the toxin molecule.

14. The fusion protein of claim **13**, wherein the second recognition molecule is different from the first recognition molecule.

15. The fusion protein of claim **13**, wherein the second recognition molecule is anti-Tac (Fv).

16. The fusion protein of claim **13**, wherein the recombinant PE molecule is TGFα-anti-Tac(Fv)-PE40.

17. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a fusion protein comprising a recombinant Pseudomonas exotoxin (PE) molecule, a first recognition moiety for binding a target cell, and a carboxyl terminal sequence of 4 to 16 residues which permits translocation of said fusion protein into the target cell cytosol, the first recognition moiety being inserted in domain III of PE after residue 600 and before residue 613.

18. The composition of claim **17**, wherein the carboxyl terminal sequence comprises, in a direction from the amino terminus to the carboxyl terminus, the following amino acid residues:

$$R^1—R^2—R^3—R^4—(R^5)_n$$

wherein,

R^1 is a positively charged amino acid residue;

R^2 is a negatively charged amino acid residue;

R^3 is a negatively charged amino acid residue;

R^4 is L; and

R^5 is a positively charged amino acid residue;

and wherein n is zero or 1.

19. The composition of claim **17**, wherein the carboxyl terminal residues are selected from the group consisting of REDLK, KEDLK, REDLR, REDL, and KDEL.

20. The fusion protein of claim **7** wherein the carboxyl terminal sequence is REDLK.

21. The fusion protein of claim 7 wherein the carboxyl terminal sequence is KEDLK.

22. The fusion protein of claim 7 wherein the carboxyl terminal sequence is REDLR.

23. The fusion protein of claim 7 wherein the carboxyl terminal sequence is REDL.

24. The fusion protein of claim 7 wherein the carboxyl terminal sequence is KDEL.

25. The fusion protein of claim 19 wherein the carboxyl terminal sequence is REDLK.

26. The composition of claim 19 wherein the carboxyl terminal sequence is KEDLK.

27. The composition of claim 19 wherein the carboxyl terminal sequence is REDLR.

28. The composition of claim 19 wherein the carboxyl terminal sequence is REDL.

29. The fusion protein of claim 19 wherein the carboxyl terminal sequence is KDEL.

* * * * *

EVIDENCE APPENDIX

PART II

EXHIBIT A

MEDLINE

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Failure of short-term CD4-PE40 infusions to reduce virus load in human immunodeficiency virus-infected persons.

Ramachandran RV, Katzenstein DA, Wood R, Batts DH, Merigan TC

J Infect Dis 1994 Oct 170:1009-13

BROWSE : J Infect Dis • Volume 170 • Issue 4

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

The safety, immunologic, and antiviral effects of a recombinant biologic product that combines the second and third domains of the CD4 molecule and Pseudomonas exotoxin A (PE40) were evaluated in 21 human immunodeficiency virus (HIV)-infected subjects in a phase III open-label dose-ranging study. Subjects with CD4+ lymphocyte counts of 100-500/mm3 received CD4-PE40 at 40, 80, or 160 micrograms/m2 by infusion three to seven times over 10 days. At the maximum tolerated dose (80 micrograms/m2), peak CD4-PE40 levels were 65-130 ng/mL with a serum half-life of 3.6 +/- 1.5 h. Toxicity, primarily increased hepatic transaminases, was dose-related and reversible. HIV DNA proviral levels in peripheral blood mononuclear cells and plasma HIV RNA remained stable during and after CD4-PE40 infusions. The relative resistance of clinical isolates of HIV, limits of the tolerated dose, and the immunogenicity and short half-life of the protein may explain the lack of in vivo antiviral effect of CD4-PE40.

MeSH

Adult; Antigens, CD4; CD4-Positive T-Lymphocytes; DNA, Viral; Drug Administration Schedule; Exotoxins; HIV; HIV Seropositivity; Human; Immunotoxins; Infusions, Intravenous; Proviruses; Pseudomonas aeruginosa; RNA, Viral; Support, Non-U.S. Gov't; Treatment Failure

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EVIDENCE APPENDIX

PART II

EXHIBIT B

Medline

Use of recombinant soluble CD4 Pseudomonas exotoxin, a novel immunotoxin, for treatment of persons infected with human immunodeficiency virus.

Davey RT, Boenning CM, Herpin BR, Batts DH, Metcalf JA, Wathen L, Cox SR, Polis MA, Kovacs JA, Falloon J

J Infect Dis 1994 Nov 170:1180-8

BROWSE : J Infect Dis • Volume 170 • Issue 5

VIEW : **MEDLINE**, full MEDLINE, related records

Abstract

Single and multiple doses of sCD4-PE40, a soluble recombinant fusion toxin selectively toxic to gp120-expressing cells, were evaluated in persons infected with human immunodeficiency virus type 1 (HIV-1). Seventeen of 24 patients who completed a single-dose safety trial were given either 1, 5, 10, or 15 micrograms/kg of sCD4-PE40 by intravenous bolus once a month for 2 months, then weekly for 6 weeks. The weekly maximally tolerated dose was 10 micrograms/kg. The major toxicity was a transient dose-dependent elevation in hepatic aminotransferases peaking 48 h after infusion. Anti-Pseudomonas exotoxin antibody developed in 58% of recipients, and sera from 13 of 17 showed neutralizing activity against sCD4-PE40. No consistent changes in immunologic or virologic markers were observed. Weekly infusions of < or = 10 micrograms/kg of sCD4-PE40 are generally well tolerated, but additional studies correlating optimal dosing and frequency of administration with efficacy will be needed to define the role of this novel agent in the management of HIV-1-infected patients.

MeSH

Adolescence; Adult; Antigens, CD4; Antiviral Agents; Exotoxins; HIV Infections; Human; Immunotoxins; Middle Age; Recombinant Proteins; Single-Blind Method; Support, Non-U.S. Gov't; Support, U.S. Gov't, P.H.S.

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EVIDENCE APPENDIX

PART II

EXHIBIT C

Proc. Natl. Acad. Sci. USA
Vol. 95, pp. 11511–11513, September 1998

Perspective

Reconsidering targeted toxins to eliminate HIV infection: You gotta have HAART

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Edited by Anthony S. Fauci, National Institute of Allergy and Infectious Diseases, Bethesda, MD, and approved August 12, 1998 (received for review June 16, 1998)

ABSTRACT **The success of highly active anti-retroviral therapy (HAART) has inspired new concepts for eliminating HIV from infected individuals. A major obstacle is the persistence of long-lived reservoirs of latently infected cells that might become activated at some time after cessation of therapy. We propose that, in the context of treatment strategies to deliberately activate and eliminate these reservoirs, hybrid toxins targeted to kill HIV-infected cells be reconsidered in combination with HAART. Such combinations might also prove valuable in protocols aimed at preventing mother-to-child transmission and establishment of infection immediately after exposure to HIV. We suggest experimental approaches *in vitro* and in animal models to test various issues related to safety and efficacy of this concept.**

Highly active anti-retroviral therapy (HAART), involving combination treatment with drugs that block different steps in the viral replication cycle (e.g., reverse transcriptase inhibitors plus protease inhibitors), has improved dramatically the health of many individuals infected with HIV (1). Despite these advances, recent analyses of peripheral blood and lymph nodes have revealed the presence of reservoirs of resting $CD4^+$ memory T cells harboring latent replication-competent provirus (refs. 2–8; reviewed in refs. 9–11). Although such reservoirs contain exceedingly small numbers of cells, they are generated very early after primary infection and persist with no significant change after 2 years of HAART. The latently infected cells are likely to activate spontaneously at some point after termination of HAART and therefore are considered to be a major obstacle to eradication of HIV from the body. This awareness has engendered the notion of deliberately "flushing out" the reservoirs by treating HAART patients with agents that activate virus expression from latently infected cells (10–12). The idea is that the virions produced on activation will be prevented by HAART from infecting new cells; it is presumed that the newly activated cells then will be eliminated by natural mechanisms such as the cytopathic effect of the virus, immune effector mechanisms, etc. (9–11). We propose that, in considering such strategies, these natural elimination mechanisms can be accelerated aggressively by using targeted toxins that selectively kill activated HIV-infected cells. Such agents may also be useful components of cocktails aimed at preventing establishment of infection in newly exposed individuals.

Hybrid Toxins Targeted to HIV-Infected Cells

During the past decade, several types of anti-HIV hybrid protein toxins have been produced by molecular genetic and biochemical methodologies (13–15). In each case, the hybrid protein contains a binding domain that targets the agent to the HIV envelope glycoprotein (Env) expressed on the surface of the infected cell and a cytotoxic domain that actively kills the cell on internalization. The hybrid toxins are constructed by substituting the normal cell binding region of the native toxin with an Env-binding domain. The Env-binding moieties used have included the extracellular regions of CD4 as well as Fab regions of anti-Env antibodies (directed against either the external subunit gp120 or the transmembrane subunit gp41). The cytotoxic domains have been derived from natural protein toxins such as *Pseudomonas aeruginosa* exotoxin A (PE), ricin, and diphtheria toxin.

To date, only one of these hybrid toxins has been tested in humans: the genetically engineered single chain protein CD4-PE40 (soluble CD4 linked to the translocation and cell killing domains of PE). For this reason, we focus on this agent, though many of our arguments also apply to other Env-targeted hybrid toxins. CD4-PE40 displays the following properties *in vitro*: cytotoxic activity against cells expressing Envs of HIV-1, HIV-2 and simian immunodeficiency virus (SIV) (16–19), high potency and specificity for killing HIV-1-infected cells with negligible effects on major histocompatibility complex Class II-expressing cells (16, 18), requirement for HIV-1 induction in a latently infected cell line (18), suppression of spreading HIV-1 infection in an acutely infected T cell line (17) and in cultures of primary T lymphocytes or macrophages (20–22), highly synergistic activity with reverse transcriptase inhibitors (23), and potent activity against primary HIV-1 strains, including those resistant to neutralization by soluble CD4 (21, 22). These *in vitro* properties, coupled with acceptable toxicity and pharmacokinetic profiles in animal studies, supported testing this agent in HIV-infected people.

Disappointing Results in Phase 1 Clinical Trials

The high hopes from the promising preclinical findings were dashed in the initial Phase I trials with HIV-infected patients (24, 25). The toxin produced dose-limiting hepatotoxicity; at the low doses that were tolerated (10 μg/kg), the peak plasma levels of CD4-PE40 remained below concentrations shown to be efficacious *in vitro*. The significant but reversible hepatotoxicity greatly diminished enthusiasm for CD4-PE40 in par-

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Abbreviations: HAART, highly active anti-retroviral therapy; Env, HIV envelope glycoprotein; PE, *Pseudomonas aeruginosa* exotoxin A; SIV, simian immunodeficiency virus; CD4-PE40, soluble CD4 linked to the effector domains of *Pseudomonas aeruginosa* exotoxin A.

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ticular and for Env-targeted toxins in general. The CD4-PE40 clinical program was terminated.

A New Context Suggests a New Concept: Testable Hypotheses

We propose that recent developments with HAART present new opportunities for exploring the therapeutic utility of Env-targeted hybrid toxins to help eradicate residual HIV-infected cell reservoirs. We hypothesize a plausible mechanism for the CD4-PE40 hepatotoxicity observed in HIV-infected people and suggest that this problem may not occur in patients with the very low viral loads achieved by HAART. We present rationales based on *in vitro* data suggesting that Env-targeted toxins might accelerate the elimination of infected cell reservoirs beyond the rates caused by natural mechanisms. These agents may also be useful components of drug cocktails aimed at preventing postexposure infection and mother-to-child transmission. Of most importance, many of these notions are subject to experimental testing *in vitro* and in animal models.

The first issue concerns the hepatotoxicity with low doses of CD4-PE40 observed in Phase I clinical trials. This problem was unexpected because preclinical toxicity studies in rodents and monkeys indicated much higher tolerated doses. Furthermore, it is now clear that hepatotoxicity is not a general property of PE derivatives in humans; several anticancer clinical trials conducted with PE-based immunotoxins have revealed striking antitumor responses without hepatotoxicity (26). Why, then, was hepatotoxicity encountered with low doses of CD4-PE40 in clinical trials with HIV-infected people? We propose that the HIV infection, particularly the high virus load, is the culprit. HIV-infected individuals likely produce free gp120 that is shed from virions or infected cells. Shedding of gp120 has been studied extensively *in vitro*; furthermore, evidence for free gp120 in sera of infected individuals has been reported, although precise measurements are confounded by the formation of immune complexes as well as by the association of released gp120 with circulating $CD4^+$ T lymphocytes (reviewed in refs. 27–29). We hypothesize that, in HIV-infected patients treated with CD4-PE40, some of the chimeric toxin associates with shed gp120. Because gp120 is glycosylated extensively and contains highly diverse oligosaccharide chains (30), the complex likely would be a substrate for the human hepatocyte asialoglycoprotein receptor, which internalizes glycoproteins containing terminal galactose or N-acetylglucosamine residues (31). The result would be the serious side effect of hepatocyte killing. Moreover, gp120/CD4-PE40 complexes bound to anti-gp120 antibodies might also contribute to liver damage.

According to this hypothesis, hepatotoxicity should not be a major problem in HAART patients because the low viral loads presumably would produce minimal amounts of free gp120. Even on induction of virus expression from latently infected memory T lymphocytes, the newly produced free gp120 is unlikely to be problematic because the number of such cells is much smaller than the number of virus-producing T lymphocytes in patients before HAART (ref. 3; also T.-W. Chun, personal communication); moreover, the newly produced gp120 will have accumulated only during the relatively short period after induction, in contrast with the prolonged duration of gp120 production before therapy. We also suggest that gp120-mediated toxicity would not be problematic when given along with HAART to newly exposed individuals because they should not yet have produced significant amounts of free gp120.

To test this model of CD4-PE40-mediated hepatotoxicity, we propose that effects of the agent be compared in animals with high vs. low levels of free gp120; we predict that hepatotoxicity will be much less severe in the latter case. There are several experimental paradigms in which this question can be examined, including comparison of CD4-PE40 hepatotoxicity in uninfected vs. chronically HIV-infected severe combined immunodeficient-hu mice or SIV-infected rhesus macaques. Perhaps more important is to use these systems to compare animals with the normal high viral loads occurring during chronic infection vs. the reduced loads achieved with potent antiviral therapy, e.g., HAART in HIV-infected severe combined immunodeficient-hu mice (32) or reverse transcriptase inhibitor therapy in SIV-infected macaques (33). A related analysis would compare in chronically infected animals the effects of hybrid toxins targeted to gp120 (e.g., CD4-PE40 and gp120-targeted immunotoxins) versus those targeted to gp41; according to our model, the latter agents would not produce hepatotoxicity even in animals with high virus load because gp41 is not released spontaneously from the membrane. In another approach, uninfected animals can be given CD4-PE40 without or with soluble gp120 to test directly whether hepatotoxicity depends on both proteins. Together, these experiments should provide important insights into whether the hepatotoxicity of CD4-PE40 is associated with high viral load, and in particular with free gp120.

HAART therapy also provides opportunities to test the therapeutic potential of Env-targeted hybrid toxins to eradicate residual infected cells. The idea is to augment their natural rates of decay, which are presumed to reflect the viral cytopathic effect and host effector mechanisms (9–11). Several previous *in vitro* studies are promising in this regard. CD4-PE40 (but not soluble CD4) markedly inhibited the spread of infection in various target cell types (17, 20–23), including primary T lymphocytes and macrophages acutely infected with primary HIV-1 strains; the interpretation of these findings is that the toxin accelerates the killing of infected cells beyond the rates associated with the viral cytopathic effect. The results with macrophages are particularly striking because these cells are refractory to HIV-mediated killing during productive infection and are thought to represent an important viral reservoir with markedly slower decay kinetics compared with $CD4^+$ T lymphocytes (9). Also of note are the promising *in vitro* results indicating highly synergistic effects of CD4-PE40 and reverse transcriptase inhibitors (23). CD4-PE40 plus 3′-azido-3′-dideoxythymidine or 2′,3′-dideoxyinosine completely inhibited acute virus replication and prevented virus-mediated killing of the $CD4^+$ target T cell population; moreover, continuation of the culture after cessation of drug treatment indicated that the infection had been eliminated completely. By contrast, each agent alone suppressed virus replication during the treatment period, but the protective effects were reversed on drug removal. These results highlight the potential value of combination treatment involving a drug(s) that inhibits HIV replication plus another that selectively kills the infected cells. Taken together, these earlier studies provide impetus for considering Env-targeted toxins to augment HAART, particularly in the context of protocols to deliberately activate virus production from latently infected cell reservoirs. These agents, in combination with other antiretrovirals, may also diminish the frequency of postexposure infection and mother-to-child transmission.

We propose additional lines of *in vitro* and *in vivo* study. Experiments can be designed to optimize the *ex vivo* activation of latently infected T lymphocytes obtained from HAART patients and to test *ex vivo* whether an Env-targeted toxin in combination with continued HAART promotes or accelerates killing of the activated cells (similar to the studies noted above with acutely infected T cells). As an *in vivo* parallel to the previous *in vitro* success with combination treatment, we propose examination of the combined effects of HAART and Env-targeted toxins in HIV-infected severe combined immunodeficient-hu mice or SIV-infected macaques. Regarding efforts to deliberately activate latently infected cells, *in vitro* experiments would guide the choice of the most promising

modes of activation. Such experiments would suggest whether Env-targeted toxins in the presence of HAART can eradicate virus from infected animals. Finally, the ability of the targeted toxins to augment other antiretrovirals in preventing infection can be examined in the HIV/severe combined immunodeficient-hu mouse and the SIV/macaque models. Favorable results in these *in vitro* and *in vivo* systems would set the stage for safety and efficacy trials of Env-targeted toxins as components of therapeutic and prophylactic protocols against HIV.

This work was funded in part by the National Institutes of Health Intramural AIDS Targeted Antiviral Program.

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EVIDENCE APPENDIX

PART II

EXHIBIT D

Chimeric Toxins Targeted to the Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Augment the In Vivo Activity of Combination Antiretroviral Therapy in thy/liv-SCID-Hu Mice

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Highly active antiretroviral therapy (HAART), which combines multiple inhibitors of essential human immunodeficiency virus type 1 (HIV-1) enzymes, induces dramatic and sustained viral load reductions in many people infected with HIV-1. However, reservoirs of infected cells capable of producing replication-competent virus persist even after years of HAART, preventing elimination of infection. CD4-PE40 and 3B3(Fv)-PE38, chimeric toxins designed to target the HIV envelope (Env), represent a complementary class of agents that selectively kill productively infected cells. To investigate whether these Env-targeted toxins might serve as adjuncts to HAART for the elimination of infected cells, we tested their ability to augment HAART efficacy in vivo by using a thy/liv SCID-hu mouse model. CD4-PE40 and 3B3(Fv)-PE38 markedly enhanced the capacity of HAART to suppress acute HIV-1 infection and improved HAART-mediated viral load reduction in mice with established HIV-1 infection. These results represent the first demonstration of in vivo anti–HIV-1 efficacy for Env-targeted toxins and support their potential therapeutic utility in combination with HAART.

Highly active antiretroviral therapy (HAART), which combines multiple inhibitors of essential enzymes of human immunodeficiency virus type 1 (HIV-1), promotes dramatic and sustained viral load reductions in plasma and tissues of many HIV-1–infected persons [1]. The associated clinical improvements and reduced death rates have transformed perspectives on HIV-1 treatment. However, it has become clear that HAART does not eradicate HIV-1 infection from the body. Reservoirs of cells harboring replication-competent virus persist in blood, lymphoid tissue, and the male genital tract even after years of treatment [2–7]; moreover, low level HIV-1 replication continues in the face of suppressive HAART [4, 8–16]. These persistent virus sources are presumed to make major contributions to the rapid HIV-1 rebound observed after cessation of therapy [17–20] and are therefore considered the major barriers to eliminating infection.

Hybrid protein toxins designed to target the HIV Env glycoprotein on the surface of productively infected cells are a complementary class of anti-HIV agents [21]. Their potent activities result not from blocking of virus replication, but from selective killing of infected cells. Such proteins contain an Env-binding moiety such as CD4 or an anti-Env antibody, linked to the effector domains of a protein toxin such as *Pseudomonas* exotoxin A (PE), ricin, or diphtheria toxin. To date, only 1 Env-targeted toxin has been tested in human clinical trials: a recombinant protein designated CD4-PE40 that contains a region of CD4 linked to the translocation and cytotoxic moieties of PE [22]. Early studies in vitro indicated highly desirable anti-HIV-1 properties, alone and in combination with reverse transcriptase inhibitors (see [23] for original citations). However, disappointing results from phase I trials conducted in the pre-HAART era [24, 25] caused this approach to be abandoned. With the subsequent development of HAART and the awareness of the need to eliminate the long-lived infected cell res-

Received 8 September 1999; revised 8 December 1999; electronically published 20 March 2000.

Presented in part: The Keystone Symposium on AIDS Pathogenesis, January 1999, Keystone, CO.

Informed consent was obtained from patients. This study followed the human experimentation guidelines of the US Departments of Health and Human Services and the Albert Einstein College of Medicine in the conduct of clinical research and followed the animal experimentation guidelines of the Albert Einstein College of Medicine in animal studies.

Financial support: National Institutes of Health (NIH; National Institute of Neurological Disorders and Stroke NS39201 and Centers for AIDS Research Grant AI-27741) and by the NIH Intramural AIDS Targeted Antiviral Program. M.P.M. was supported by an NIH training grant (CA09173).

Some of the authors of the manuscript either have patents or patents pending on the Env-targeted toxins described in the paper. E.A.B. is an inventor on US-owned patents related to CD4-PE40 and on US patent application for 3B3(Fv)-PE38. I.P. is an inventor on patents related to PE technology, on US-owned patents related to CD4-PE40, and on US patent application for 3B3(Fv)-PE38. T.B. is an inventor on US patent application for 3B3(Fv)-PE38. We do not feel that their patent position poses a conflict of interest for the findings presented in this paper.

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The Journal of Infectious Diseases 2000; 181:921–6

0022-1899/2000/18103-0015$02.00

ervoirs, we recently proposed that the specific cytotoxic activity of Env-targeted toxins against HIV-1–infected cells warrants renewed consideration [23]. We suggested various experimental approaches, both in vitro and in vivo, to test the efficacy and safety of these agents when used in conjunction with HAART. Presently there are no published data showing the in vivo efficacy of Env-target toxins, either alone or in combination with other antiretrovirals.

In the present study, we evaluated the capacity of Env-target toxins to enhance HAART efficacy in vivo in a mouse model. We used SCID mice implanted with human fetal thymus and liver tissue under the kidney capsule (thy/liv-SCID-hu mice). We have previously shown that this model is well suited for evaluating the efficacy of different regimens of HAART in vivo, in both prevention and treatment modes [26]. Two distinct chimeric toxins were tested: CD4-PE40 and 3B3(Fv)-PE38 [27], which is a potent immunotoxin containing the effector portions of PE linked to a single-chain Fv from an affinity-enhanced, broadly cross-reactive antibody against the CD4-binding region of gp120. Our results reveal the ability of Env-targeted toxins to significantly enhance the anti-HIV-1 activity of HAART in experimental protocols examining both the prevention of acute infection and the treatment of chronic infection.

Materials and Methods

Env-targeted toxins and control proteins. CD4-PE40 and sCD4 (first 2 extracellular domains, amino acids 1–183) were donated by Shirley Johnson at Pharmacia-Upjohn, Kalamazoo, MI. The control immunotoxin, RFB4(dsFv)-PE38, is directed at the human B cell antigen CD22 [28] and is not expected to affect HIV-1 infection. We generated sCD4 using mammalian cell expression, and the chimeric toxins were produced using bacterial expression [22, 27].

Implantation of human thymic and liver tissue into SCID mice. The thy/liv-SCID-hu mice were constructed by implanting human fetal thymic and liver tissue obtained from 17–21-gestational-week fetuses within 8 h after the elective termination of pregnancy into SCID mice (6–8 weeks old) as described elsewhere [26]. Briefly, about 10 pieces of syngeneic human fetal thymic and liver tissue were implanted under the left and right kidney capsules of SCID mice anesthetized with pentobarbital (40–80 mg/kg). The procedure was associated with minimal morbidity and mortality and was successful in >95% of the mice, as indicated by a >20-fold increase in size of the implanted tissue 3 months later. We used flow cytometry to confirm the population of human T cells and monocytes in the peripheral blood of these mice; in the mice used for these experiments, the peripheral blood contained >5% human leukocytes. The consent forms and procedures used in this study were reviewed and approved by the Albert Einstein College of Medicine Committee on Clinical Investigation.

Infection of thy/liv-SCID-hu mice with HIV. HIV-1_{59}, an R5 (CCR5-specific, nonsyncytium-inducing) primary isolate, was derived from peripheral blood mononuclear cells (PBMC) by coculture with phytohemagglutinin (PHA)-activated donor PBMC [29] from a 17-month-old child infected with HIV-1; the virus was expanded by another round of coculture with PHA-activated PBMC and then divided into aliquots that were frozen in liquid nitrogen [26, 29]. thy/liv-SCID-hu mice were infected by intraperitoneal (ip) injection of 8000 $TCID_{50}$ of the HIV-1_{59} in a volume of 500 μL. To control for variability due to the source of donor tissue and degree of reconstitution with human cells, we ensured that each treatment group contained mice engrafted with the same donor tissue and with similar quantities of human cells in their peripheral blood.

Administration of HAART and Env-targeted toxins. We administered HAART to the mice by mixing the indicated drugs with powdered animal feed and then adding the mixture to feeding jars that were designed to minimize spillage. The drug dosage for each mouse was calculated on the basis of the average oral intake of 5 g of diet per day (each mouse weighed ~25 g). Mice were treated with HAART comprised of zidovudine (100 mg kg^{-1} day^{-1}), lamivudine (100 mg kg^{-1} day^{-1}), and ritonavir (200 mg kg^{-1} day^{-1}) or suboptimal HAART (10% of the dose for each HAART drug) as described elsewhere [26]. The thy/liv-SCID-hu mice were housed singly so that drug consumption could be confirmed by measurement of the quantity of feed consumed. The Env-targeted toxins were administered by ip injection of the indicated dose in PBS in 5 doses given on alternate days.

Titration of HIV-infected mononuclear cells in the hu-thy/liv implant by limiting dilution coculture. The level of HIV-1 infection in the hu-thy/liv implants was determined by measuring the number of HIV-1–infected thymocytes present in a wedge biopsy of the implant by quantitative coculture, as described elsewhere [26, 29]. Five-fold dilutions of mononuclear cells isolated from the hu-thy/liv implants (range, 1×10^6–3.2×10^2 cells) were cultured in quadruplicate at 37°C in 24-well culture plates with PHA-activated donor mononuclear cells (1×10^6) in 2 mL of RPMI 1640 containing fetal calf serum (10% v/v) and interleukin-2 (32 U/mL). The p24 antigen content of the culture supernatant was measured 1–2 weeks later by means of the HIV-1 p24 core profile ELISA assay (DuPont-NEN, Wilmington, DA). The number of HIV-1–infected thymocytes in the implants is reported as TCID/10^6 thymocytes, which was calculated by determining the lowest number of added thymocytes that infected at least half of the quadruplicate cultures with HIV-1. Productive infection after the addition of the lowest number of added thymocytes (320 cells) was scored as 3125 TCID/10^6 thymocytes.

Statistical analysis. Statistical significance of the data were evaluated by using the Student's t test.

Results

Env-targeted toxins enhance capacity of HAART to suppress acute HIV-1 infection. We have shown previously that, whereas HAART that is initiated immediately after HIV-1 inoculation of thy/liv-SCID-hu mice prevents establishment of infection, suboptimal HAART (10-fold lower doses of the same drugs) strongly suppresses HIV-1 levels during the treatment period, but does not prevent the subsequent appearance of viral load at 1 month after the cessation of treatment [26]. Here, we examined the in vivo antiretroviral effect of Env-targeted toxins by determining whether they augmented the activity of suboptimal HAART. Thy/liv-SCID-hu mice were infected by ip in-

oculation of HIV-1_{59} [29], then immediately started on the indicated treatment. After 1 month, the effects of the different treatments on HIV-1 infection were determined by quantitating HIV-1–infected thymocytes in a biopsy of the hu-thy/liv implants; this method is much more sensitive than measuring plasma HIV RNA and can quantitate viral load in HAART-treated animals that have no detectable plasma viremia [26]. The mice were then taken off therapy, and 1 month later the hu-thy/liv implants were biopsied again and analyzed.

The effects on suboptimal HAART alone or Env-targeted toxins alone are shown in figure 1. In contrast to untreated mice, in which extensive infection (mean = 2708 TCID/10^6 thymocytes) was detected in the thy/liv implants at both 1 and 2 months after virus inoculation, mice maintained on suboptimal HAART did not have detectable HIV-1 infection at the end of the 1-month treatment period (mean = 0 TCID/10^6 thymocytes). However, HIV-1 infection emerged during the 1-month period after cessation of therapy (mean = 258 TCID/10^6 thymocytes), which is consistent with our previous findings with this suboptimal HAART regimen [26]. The Env-targeted toxins alone suppressed infection only modestly compared with HAART, but the antiretroviral effects were significant (for CD4-PE40, mean = 975 TCID/10^6 thymocytes, $P = .028$; for 3B3(Fv)-PE38, mean = 292 TCID/10^6 thymocytes, $P = .003$). In contrast, the mixture of sCD4 plus the control toxin RFB4(dsFv)-PE38 did not significantly suppress HIV infection in the thy/liv implants (mean = 1875 TCID/10^6 thymocytes, $P = .15$), which indicates that the effects seen with the Env-targeted toxins resulted from selective killing of infected cells.

We next examined whether Env-targeted toxins could enhance the efficacy of suboptimal HAART in suppressing acute HIV-1 infection in the thy/liv-SCID-hu mice. As shown in table 1, the viral loads in the hu-thy/liv implants at 1 month after cessation of therapy remained markedly suppressed in the mice treated with the combination of lower-dose HAART plus either Env-targeted toxin, in contrast to the mice treated with HAART alone, in which the viral load increased significantly. Again, the control proteins sCD4 plus RFB4(dsFv)-PE38 did not augment the anti-HIV-1 activity of HAART, which verified that the activities seen with the Env-targeted toxins reflected specific killing of infected cells, not simple neutralization by the CD4 moiety or nonspecific toxicity by the PE moiety. Thus, by virtue of their specific cytotoxic activities, the Env-targeted toxins administered immediately after HIV-1 inoculation greatly enhanced the capacity of suboptimal HAART to suppress acute infection.

Env-targeted toxins enhance the capacity of HAART to treat established HIV-1 infection. We have shown previously that HAART significantly decreases the level of infection in the hu-thy/liv implants of thy/liv SCID-hu mice with established HIV-1 infection, but does not eradicate it, as evidenced by the moderate numbers of residual HIV-1–infected cells that remain in the thymic implants [26]. Therefore, we also used the thy/liv-SCID-hu mouse system to examine the ability of Env-targeted toxins to augment HAART for treating established in vivo HIV-1 infection. One month after ip inoculation with HIV-1_{59}, the baseline level of HIV-1 infection was determined by quantitative coculture of biopsies from the hu-thy/liv implants; the values were 3125 $TCID_{50}/10^6$ thymocytes for each of the infected mice. The mice were then treated with HAART alone, HAART plus CD4-PE40, or HAART plus 3B3(Fv)-PE38. After 1 month of treatment, the effects of these therapeutic regimens on HIV infection in the treated mice were determined by quantitative coculture of repeat biopsies of the hu-thy/liv implants. In the untreated mice, the levels of infection in the implants did not decrease, remaining at 3125 $TCID_{50}/10^6$ thymocytes. By contrast, as shown in table 2, the viral loads in the thy/liv implant decreased significantly in the mice treated with HAART alone ($P < .001$), HAART plus CD4-PE40 ($P < .001$), or HAART plus 3B3(Fv)-PE38 ($P < .001$). With HAART plus 3B3(Fv)-PE38,

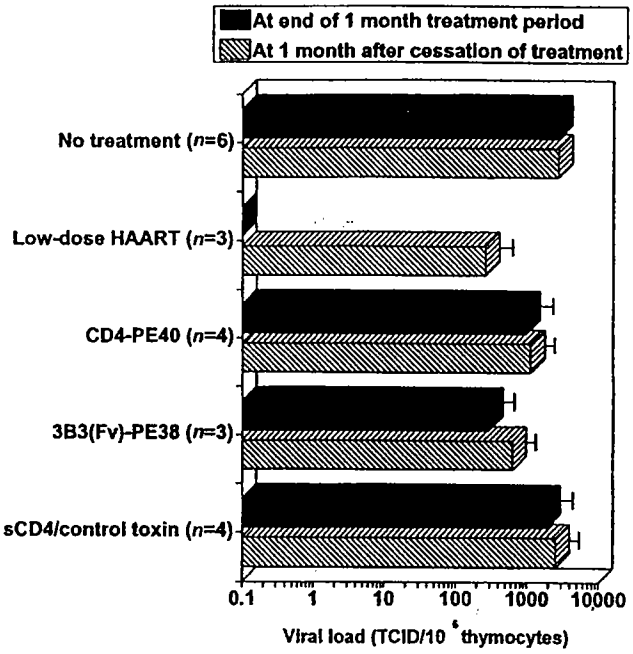

Figure 1. Effects of suboptimal highly active antiretroviral therapy (HAART) or human immunodeficiency virus (HIV) envelope-targeted toxins on acute HIV-1 infection in vivo. thy/liv-SCID-hu mice were injected intraperitoneally with HIV-1_{59} (8000 $TCID_{50}$). The mice were then left untreated or were immediately started either on suboptimal HAART, CD4-PE40, 3B3(Fv)-PE38, or a control mixture of sCD4 plus RFB4(dsFv)-PE38 (control toxin); toxins were administered at a total dose of 5 μg given in 5 equal doses on alternate days beginning at day one. One month later, the left thy/liv implants were biopsied, and viral loads were analyzed by quantitative coculture *(dark bars)*. Drug therapy was then stopped, and after 1 month the left thy/liv implant of each mouse was rebiopsied and the viral load quantitated *(light bars)*. The data are presented as the TCID/10^6 thymocytes, determined by limiting dilution coculture; the mean values (±SEM) were calculated for each treated group.

Table 1. Measurements of viral load in thy/liv-SCID-hu mice infected with acute human immunodeficiency virus (HIV), showing that HIV envelope-targeted toxins enhance the ability of suboptimal highly active antiretroviral therapy (HAART) to suppress acute HIV in vivo.

Treatment (dose), mouse no.	Viral load, TCID/10^6 thymocytes	
	At end of 1-mo treatment period	1 mo after end of treatment
None		
261-a3	3125	3125
261-a4	625	625
261-b2	3125	3125
253-b5	3125	3125
259-a2	3125	3125
250-a3	3125	3125
Mean ± SEM	2708 ± 416	2708 ± 416
Suboptimal HAART		
250-b2	0	25
250-b3	0	125
253-b4	0	625
Mean ± SEM	0	258 ± 185
Suboptimal HAART plus:		
CD4-PE40 (50 ng)		
252-a4	0	0
252-a3	0	0
252-b	0	0
Mean	0	0
CD4-PE40 (5 μg)		
261-a1	0	0
261-a5	1	5
265-a3	0	1
Mean ± SEM	0.3 ± 0.2	2 ± 1.5
3B3(Fv)-PE38 (50 ng)		
253-a3	0	0
253-a4	0	0
250-a5	0	0
Mean ± SEM	0	0
3B3(Fv)-PE38 (5 μg)		
261-c2	0	1
265-a4	0	1
Mean ± SEM	0	1 ± 0
sCD4 + control toxin (5 μg)[a]		
258-2	0	125
265-b5	0	125
265-d5	0	625
258-3	0	125
Mean ± SEM	0	250 ± 125

NOTE. The mice were injected intraperitoneally with HIV-1_{39} (8000 $TCID_{50}$) and either not treated or immediately started on treatment. Dose amounts are the total amount of drug administered in 5 equal doses on alternate days beginning at day 1.

[a] RFB4(dsFv)-PE38 (5 μg), a negative control toxin not expected to affect HIV infection. The data are presented as the TCID/10^6 thymocytes, determined by limiting dilution coculture.

the viral load was significantly lower ($P = .04$) than with HAART alone (mean, 10.3 ± 5.7 vs. 91.7 ± 26 TCID/10^6 thymocytes). This demonstrates that 3B3(Fv)-PE38 can augment the antiretroviral activity of HAART for the treatment of established in vivo HIV-1 infection. The viral loads in mice treated with HAART plus CD4-PE40 were also lower than those treated with HAART alone (mean, 51.7 ± 28.7 vs. 91.7 ± 26), but the difference was not statistically significant ($P = .23$). The increased in vivo activity of 3b3(Fv)-PE38 is consistent with the results of previous in vitro studies, which indicates that 3b3(Fv)-PE38 has a somewhat greater potency than CD4-PE40 [27].

Discussion

The findings of this study are the first demonstration that Env-targeted toxins have anti-HIV-1 activity in vivo. They also validate the utility of our thy/liv SCID/hu mouse system for evaluating the in vivo efficacy of different anti-HIV multidrug regimens. In particular, the emergence of HIV infection after the cessation of HAART in these mice provides an in vivo system that can be used to examine the additive anti-HIV-1 effects of various agents used in conjunction with HAART. The activities of the toxins were observed in models for both acute and established infection. However, although the toxins had prominent activity when used in conjunction with HAART, they were minimally effective when used alone. These results are consistent with 2 of our earlier findings in vitro [23, 30]: (1) CD4-PE40 only partially blocks spreading HIV infection, and (2) CD4-PE40 displays potent synergistic activity with a reverse transcriptase inhibitor; combination treatment completely eliminates infectious HIV-1 from cell cultures, an effect that was not achievable with either agent alone. The modest in vitro and in vivo effects observed with the Env-targeted toxins alone can be simply explained by their known mechanism of action; these agents can exert their anti-HIV activities only after Env has been expressed on the surface of the infected cell. Thus, complete suppression of virus spread would be expected only if the cells are killed before any progeny virions are released. Given the complex kinetic parameters governing the rates of cell killing versus virion release, it is not surprising that the toxins alone fail to completely eliminate the infected cell population before some viral spread occurs. Complementary effects might be anticipated when the toxins are combined with inhibitors of essential HIV replication enzymes because the latter agents effectively block subsequent rounds of infection by newly released virions but do not attack cells that are already infected. In the present in vivo experiments, suboptimal HAART was strongly augmented by CD4-PE40, but not by the control mixture of sCD4 plus an irrelevant PE-based toxin. This finding verifies that the complementary activity of the Env-targeted toxin was indeed due to selective killing of the HIV-infected cells. However, we cannot rule out the possibility that other anti-HIV agents with entirely different modes of action might also show complementary activities with the suboptimal HAART regimen.

The present studies are the beginning of a new effort to assess the clinical potential of complementing HAART with Env-targeted toxins, as proposed elsewhere [23]. The toxins used could be those examined in this report or similar agents developed by other investigators [21]. This modality could possibly be used

Table 2. Measurements of viral load in thy/liv-SCID-hu mice infected with human immunodeficiency virus (HIV) showing that HIV envelope (Env)–targeted toxins enhance the ability of highly active antiretroviral therapy (HAART) to suppress established HIV-1 infection in vivo.

Treatment, mouse no.	Viral load, TCID/10^6 thymocytes	
	1 mo after infection, before treatment	After 1 mo of treatment
None		
253-b5	3125	3125
259-a2	3125	3125
250-a3	3125	3125
Mean ± SEM	3125 ± 0	3125 ± 0
High-dose HAART		
255-c4	3125	125
255-c5	3125	125
255-c6	3125	25
Mean ± SEM	3125 ± 0	91.7 ± 26
High-dose HAART plus:		
CD4-PE40		
255-c2	3125	25
255-c1	3125	125
255-b6	3125	5
Mean ± SEM	3125 ± 0	51.7 ± 28.7
3B3(Fv)-PE38		
255-b2	3125	5
255-a4	3125	25
255-a3	3125	1
Mean ± SEM	3125 ± 0	10.33 ± 5.7

NOTE. The mice were injected intraperitoneally with HIV-1_{89} (8000 $TCID_{50}$). Env-target toxin was administered at a total dose of 50 ng, given in 5 doses on alternate days starting 3 weeks after the initiation of HAART.

to prevent the establishment of persistent HIV-1 infection in individuals presenting soon after HIV-1 exposure (e.g., newborns of infected mothers; postexposure prophylaxis). Moreover, Env-targeted toxins may serve to help eliminate reservoirs of infected cells that persist after years of HAART; these reservoirs include latently infected resting $CD4^+$ memory T cells in the circulation and lymphoid tissue [26], blood and lymphoid cells responsible for the virus replication that persists after HAART [4, 8–16], and perhaps other cell types and tissue compartments that are refractory to HAART [7].

Latently infected cells are a particularly difficult problem. It has been proposed that the latent reservoirs may be flushed out by deliberate treatment with agents that activate latently infected cells [4]. Indeed, supplementing HAART with intermittent IL-2 therapy has been shown to significantly reduce the pool of latently infected resting $CD4^+$ T cells in blood and lymph nodes [31]. However, the IL-2 treatment had negligible effect on the viral load rebound after cessation of therapy [19]. We propose that in such protocols, the Env-targeted toxins may effectively kill the newly activated cells, because these cells should be induced to express surface Env. However, we note that the toxins will be beneficial only if they accelerate the natural mechanisms governing the decline of persistent virus-producing cells (i.e., intrinsic cellular decay rates, viral cytopathic effects, and immune effector mechanisms such as cytotoxic T cells).

Another critical issue for using Env-targeted toxins in conjunction with HAART is the dose-limiting hepatotoxicity observed with CD4-PE40 in earlier phase I trials. This problem is perplexing for several reasons. First, dose-limiting hepatotoxicity was observed in HIV-1–infected people (maximum tolerated dose [MTD] 10 μg/kg, [24, 25]) but was not seen in preclinical toxicology studies with uninfected rodents and nonhuman primates (MTD 250 μg/kg; unpublished results). Second, in recent phase I trials for tumor therapy, different PE-based immunotoxins have been well tolerated, and significant antitumor activity was observed at tolerable doses (20–40 μg/kg) without serious hepatotoxicity ([32]; R. Kreitman, L. Pai-Scherf, and I. Pastan, unpublished results). Thus, the dose-limiting hepatotoxicity previously observed with CD4-PE40 appears to be unique to the use of this chimeric toxin in HIV-1–infected individuals. We have suggested [23] that this problem may have resulted from the high viral loads in subjects treated in the pre-HAART era. Specifically, we proposed that soluble gp120 shed from virions or infected cells would bind to circulating CD4-PE40; the resulting complexes would be delivered to the liver by several mechanisms. If this notion is valid, then hepatotoxicity should not pose a problem under conditions of HAART-mediated virus suppression. Alternatively, if the hepatotoxicity is a unique feature of CD4-PE40 in humans, then immunotoxins such as 3b3(Fv)-PE38 should not be problematic, particularly in view of the absence of severe hepatotoxicity with other PE-based antitumor immunotoxins in phase I trials, as noted earlier. The in vivo results presented here provide an impetus for diverse lines of experimentation, both for in vitro and animal models, to evaluate these critical issues. Such efforts may help pave the way for effective clinical efforts to eradicate HIV-1 infection from the body.

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EVIDENCE APPENDIX

PART II

EXHIBIT E

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: PASTAN et al. Application No.: 09/673,707 Filed: January 11, 2001 For: RECOMBINANT IMMUNOTOXIN DIRECTED AGAINST THE HIV-1 GP120 ENVELOPE GLYCOPROTEIN Customer No.: 20350	Confirmation No. 3958 Examiner: Zeman, Robert A. Technology Center/Art Unit: 1645 DECLARATION OF DR. DAVID J. FITZGERALD

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, DR. DAVID J. FITZGERALD, hereby declare and state:

1. I received a B.A. in Microbiology from Trinity College, Dublin, Ireland, in 1977, and a Ph.D. in Microbiology from the University of Cincinnati, College of Medicine, Cincinnati, Ohio, in 1982.

2. Since receiving my doctorate in 1982, I have been a researcher in the Laboratory of Molecular Biology ("LMB") of the National Cancer Institute ("NCI"), of the U.S. National Institutes of Health ("NIH"). Since 1994, I have been Chief of the LMB's Biotherapy Section.

3. I am not an inventor on the captioned patent application, have no financial interest in it, and do not expect that any aspect of my employment at NIH will be affected by my submission of this Declaration.

4. I am an author or co-author of over 180 publications in the scientific literature. A copy of my c.v. is attached as Attachment 1.

5. I have been working in the field of targeting toxins to target cells since 1982. As reflected by the publications listed on my c.v., I have extensive experience in targeting toxins to target cells by attaching the toxins to targeting agents including (i) antibodies or fragments thereof (these hybrid molecule are known as "immunotoxins"), (ii) cytokines, and (iii) other molecules, including CD4, that are capable of targeting toxins to desired cell types. I have performed studies on aspects of each of these types of targeted toxins, and followed closely the results of using targeted toxins in clinical trials.

6. I am specifically knowledgeable about the attempts to develop targeted toxins of CD4-*Pseudomonas* exotoxin A ("PE") for use as therapeutic agents for HIV-1 infection, as reflected by the fact that I was a co-author on the first study on the use of a CD4-PE chimeric toxin to kill HIV-1 infected cells:

Chaudhary, V.K., Mizukami, T., Fuerst, T.R., **FitzGerald, D.J.**, Moss, B., Pastan, I., and Berger, E.A., "Selective killing of HIV-infected cells by recombinant human CD4-*Pseudomonas* exotoxin hybrid protein." Nature 335:369-372 (1988). A copy of the abstract of this publication is attached as Attachment 2 hereto.

7. My work in the pre-clinical development of CD4-PE toxin conjugates as a therapeutic to treat HIV-1 disease is further reflected by my co-authorship on the following publications:

i.) Berger, E.A., Clouse, K.A., Chaudhary, V.K., Chakrabarti, S., **FitzGerald, D.J.**, Pastan, I., and Moss, B., "CD4-*Pseudomonas* exotoxin hybrid protein blocks the spread of human immunodeficiency virus infection *in vitro* and is active against cells expressing the envelope glycoproteins from diverse primate immunodeficiency retroviruses. Proc. Natl. Acad. Sci. USA 86:9539-9543 (1989) A copy of the abstract of this publication is attached as Attachment 3 hereto.

ii.) Moss, B., Mizukami, T., Fuerst, T., Berger, E., Chaudhary, V., **FitzGerald, D.**, and Pastan, I., Localization of the HIV-binding region of CD4 and selective killing of HIV-infected cells with a hybrid CD4-*Pseudomonas* exotoxin. In: Girard, M. and

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iii.) Berger, E.A., Chaudhary, V.K., Clouse, K.A., **FitzGerald, D.J.**, Pastan, I., and Moss, B.:"Recombinant CD4-*Pseudomonas* exotoxin hybrid protein: Specific cytotoxic activity against T-cell lines infected with human immunodeficiency virus. In Groopman, J.E., Chen, I., Essex, M., and Weiss, R. (Eds.): Human Retroviruses, UCLA Symposia on Molecular and Cellular Biology, New Series, Vol 119. New York, Alan R. Liss, Inc., 1989, pp. 261-270.

iv.) Ashorn, P., Moss, B., Weinstein, J.N., Chaudhary, V.K., **FitzGerald, D.J.**, Pastan, I., and Berger, E.A, "Elimination of infectious HIV from human T-cell cultures by synergistic action of CD4-*pseudomonas* exotoxin and reverse transcriptase inhibitors." Proc. Natl. Acad. Sci. USA 87: 8889-8893 (1990) A copy of the abstract of this publication is attached as Attachment 4 hereto.

v.) Berger, E.A., Chaudhary, V.K., Clouse, K.A., Taraquemada, D., Nicholas, J.A., Rubino, K.L., **FitzGerald, D.J.**, Pastan, I., and Moss, B.: Recombinant CD4-*Pseudomonas* exotoxin hybrid protein displays HIV-specific cytotoxicity without affecting MHC class II-dependent functions. AIDS Res. Hum. Retroviruses 6: 795-804, 1990. A copy of the abstract of this publication is attached as Attachment 5 hereto.

vi.) Chaudhary, V.K., Moss, B., Berger, E.A., **FitzGerald, D.J.**, and Pastan, I.: CD4-PE40: A chimeric toxin active against HIV-infected cells. In Gallo, R.C. and Jay, G. (Eds.): The Human Retroviruses. Orlando, FL, Academic Press, 1991, pp. 379-387.

8. I followed closely the clinical trials of CD4-PE chimeric toxins. I am familiar with the results of those trials and with what persons of skill in the art believed following the failure of these chimeric toxins in those trials.

9. I am also familiar with the anti-gp120 antibody known as 3B3, as described in the specification of the captioned application. I am aware of the binding specificity and affinity of the Fv portion of this antibody and of the results of using immunotoxins composed of fusing the Fv portion of 3B3 (hereafter referred to as "3B3 Fv") to PE to kill cells infected with HIV-1.

10. I understand that the claims currently under examination in the captioned application are directed to immunotoxins having the binding specificity of 3B3 Fv and the binding affinity of 3B3 Fv, kits containing such immunotoxins, and compositions of these antibodies and a pharmaceutically acceptable carrier.

11. I understand that the Office Action dated April 13, 2006 (hereafter, "the Action"), regarding this application rejects the claims under examination as obvious over Matsushita et al., Aids Research Human Retroviruses 6(2):193-203 (1990) (hereafter, "Matsushita"), in view of Barbas, PNAS 91:3809-3813 (1994) and Pastan, U.S. Patent No. 5,458,878. I understand that the Matsushita reference relates to an anti-gp120 antibody known as 0.5β.

12. I understand that the counsel for the applicants has argued that the high hopes that might have been existed for the use of anti-gp120 antibodies at that time Matsushita was published in 1990 were dashed by the results of clinical trials of CD4-PE40 reported by Ramchandran et al., J. Infect Dis 170:1009-13 (1994) ("Ramachandran") and by Davey et al., J. Infect Dis 170:1180-8 (1994) ("Davey").

13. I understand that, on page 6, the Action states that the immunotoxin used in Ramanchandran and Davey are not analogous to the immunotoxins claimed in the claims under examination. I understand that the Action explains this conclusion as follows:

> " The immunotoxins of the instant invention . . . target cells expressing gp120 on their surface (i.e., infected cells) whereas the CD4-PE40 immunotoxin of Ramachandran et al. target any cell expressing CD4. Hence any 'results' based on the application of CD4-PE40 immunotoxin would not have any bearing on the perceived efficacy of immunotoxin based on the combination of the cited references. The same is true for the sCD[4]-PE immunotoxin disclosed by Davey et al."

(Technically, toxins targeted by non-antibody targeting molecules such as CD4 are not referred to in the art as "immunotoxins." Since the Action refers to the CD4-targeted toxins of Ramachandran and Davey as "immunotoxins," I will refer to them as such in this Declaration.)

14. The Action's position is factually untrue and would have been known to be false by a person of skill in the art as of the June 1998 filing date of the priority application. CD4 is a cell surface marker on the surface of certain cell types, B cells and macrophages that is bound by the gp120 protein of HIV-1. CD4 does not bind to itself. Neither the CD4-PE40 immunotoxin of Ramachandran nor the sCD4-PE immunotoxin of Davey would bind cells expressing CD4, as stated by the Action.

15. What the CD4-PE40 immunotoxin of Ramachandran and the sCD4-PE immunotoxin of Davey were intended to bind were cells infected by HIV-1, which express gp120 on their surface. The immunotoxins recited in the claims under examination have the binding affinity of the 3B3 Fv, which binds to the gp120 protein. Thus, both (i) the CD4-PE40 immunotoxin of Ramachandran and the sCD4-PE immunotoxin of Davey, and (ii) the immunotoxins of the present invention, bind to cells expressing gp120, not to cells expressing CD4. I and others in the art would therefore consider them to be analogous in terms of the cells they were intended to bind.

16. CD4 has interactions with major histocompatibility ("MHC") class 2 molecules. There was some intellectual concern at the time that CD4-PE toxins would bind to macrophages and other cells that express MHC class 2 molecules. This concern was tested pre-clinically, and found not be a concern well before the studies reported by Ramachandran and Davey. See, e.g., the Berger et al. (1990) publication listed above at ¶ 7, item (v).

17. I understand that, on page 6, the Action states:

"With regard to Point 4, contrary to Applicants assertion, CD4-P[E]40 immunotoxins would bind not only to cells expressing gp120, but also to any cell expressing CD4 on its surface."

This statement is factually untrue and would have been known to be false by a person of skill in the art as of the June 1998 filing date of the priority application. As already noted above, CD4 does not bind to itself. CD4-PE toxins do not bind "to any cell expressing CD4 on its surface." They do bind (and kill) cells expressing gp120 on their surface, as reported in my publications listed in paragraphs 6 and 7, above. And the only cells in the body that express gp120 are those infected with HIV-1.

17. I understand that, on page 6, the Action further states:

> "With regard to Point 5, since the CD4-PE40 immunotoxin would bind to any cell expressing CD4 on its surface, the hepatoxicity would logically be the result of said immunotoxin binding to healthy cells thereby disrupting some cellular or endocrine cascade present in man but not in the mouse."

This statement is factually untrue and would have been known to be false by a person of skill in the art as of the June 1998 filing date of the priority application. First, as already noted above, CD4 does not bind to itself. CD4-PE toxins do not bind "to any cell expressing CD4 on its surface." Second, cells in the liver (hepatocytes) do not express CD4. Thus, even if the Action was not incorrect about CD4-PE binding to CD4, the Action's argument would fail to explain the hepatoxicity observed in the human trials of CD4-PE toxins.

18. The above statements set forth my correction of the serious factual errors set forth in the Action. Not surprisingly, since the facts on which the Action are based are wrong, it also incorrectly presents what I and others of skill in the field understood following the results of the clinical trials of CD4-PE immunotoxins and before the filing of the captioned application.

19. At page 7, the Action states that the:

> "failure of a non-analogous immunotoxin [the CD4-PE immunotoxins] while it may have been discouraging would not necessarily remove the motivation provided by Matsushita, especially when his immunotoxin (which is analogous to the instant invention) was disclosed to have efficacy."

I consider this analysis to be incorrect, for several reasons.

(i) First, the CD4-targeted toxins, the 0.5β antibody of Matsushita, and the immunotoxins of the claims under examination all target cells expressing gp120, that is, HIV-1 infected cells. Matsushita's antibody is type-specific (see point (iv) below) and therefore binds only to cells infected by HIV-1 of the correct type, while both CD4 and the immunotoxins of the present invention would both bind to cells with less regard to the particular type of HIV-1 infecting the cells. But, to the extent that they are considered as binding to HIV-1 infected cells in preference to cells that are not infected by HIV-1, they would be considered analogous by persons of skill in the art.

(ii) Second, as noted above, liver cells do not express CD4. Thus, as noted above, even assuming that, contrary to fact, the CD4-PE immunotoxins would bind to cells expressing CD4, there would be no reason to think that the hepatoxicity observed in trials of CD4-PE immunotoxins would not also be found with respect to toxins targeted by the antibody of Matsushita.

(iii) Third, the Action comments that the antibody of Matsushita "was disclosed to have efficacy." The efficacy Matsushita discloses is that "toxin-conjugated anti-gp120 monoclonal antibody selectively killed HIV-infected cells in vitro." Matsushita, at page 199, second paragraph. Thus, the efficacy disclosed in Matsushita is similar to that disclosed in my publication in Nature two years earlier regarding the in vitro efficacy of CD4-PE in killing HIV-1 infected cells. See, Chaudhary et al., Nature 355:369-72 (1988). This would not by itself give persons of skill any reason to expect a different result with the 0.5β antibody of Matsushita than that found in clinical trials of CD4-PE toxins.

(iv) Fourth, Matsushita states that the 0.5β antibody is "type-specific." Matushiata, page 194, first line under heading "Antibody and immunotoxins." Matsushita notes in its discussion section that, while the binding activity of the 0.5β antibody was type specific, toxins conjugated to CD4 "also killed HIV-infected cells in vitro and were shown to be effective against [a] variety of divergent strains of HIV." Id., at page 200, second paragraph. Thus, Matsushita itself indicated the superiority of CD4 as a targeting agent against HIV-1 infected cells to the antibody the Matsushita authors themselves had developed. Any motivation Matsushita provided to create anti-env immunotoxins was removed by the failure of the CD4 toxins that the Matsushita authors themselves indicated were more broadly applicable than those the immunotoxin they had developed.

(v) Finally, both the immunotoxin of Matsushita and CD4-PE are toxins targeted to the envelope glycoprotein ("Env") of HIV-1.

For all of these reasons, even assuming that Matsushita provided a motivation to make Env-targeted toxins prior to the of the CD4-PE trials, I disagree with the Action's conclusion that Matsushita continued to provide such a motivation following the failure of those trials.

20. I note that the Action also states:

> With regard to Point 7, the "long felt need" for AIDS treatments was met by the teachings of Matsushita and would provide additional motivation for the skilled artisan to further refine the teachings of Matsushita.

Action, at page 7. I believe this statement is without merit. With respect, Matsushita's teachings did not "meet" the "long felt need" for AIDS treatments. Matsushita's 0.5β antibody-based immunotoxin is not only type-specific, but is also targeted to an epitope that even Matsushita admits is "within a highly variable region of gp120." Matsushita, at page 194, first full paragraph. Indeed, the Matsushita antibody proved unsuitable for clinical development since the site it binds is one the HIV-1 virus readily mutates so that infected cells do not express the epitope bound by the antibody. I am not aware, some 16 years after the publication of Matsushita in 1990, that the Matsushita 0.5β antibody was ever brought into pre-clinical development. It clearly did not "meet the long-felt need" for an AIDS treatment, as asserted by the Action. In contrast, 3B3Fv-targeted immunotoxins of the claims are continuing to be successful in pre-clinical studies, including one designed to see if the immunotoxin would induce the same hepatoxicity as that seen in the CD4-PE trials referenced above. See, Kennedy et al., J Leukoc Biol (August 2006). A copy of the abstract of this publication is attached as Attachment 6 hereto.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these

statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under § 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Oct 13th, 2006
Date

David J. FitzGerald
Dr. David J. FitzGerald

EVIDENCE APPENDIX

PART II

EXHIBIT E1

CURRICULUM VITAE

Name	David J. FitzGerald
Current Position	Chief, Biotherapy Section Laboratory of Molecular Biology Division of Basic Science National Cancer Institue, NIH
Address and Phone	37 Convent Dr, Bldg 37, 5124 Bethesda, MD 20892-4255 301-496-9457 ph 301-402-1969 fax djpf@helix.nih.gov

Education

Trinity College, Dublin, Ireland BA Mod 1977 Microbiology
U of Cincinnati, Col of Med, OHPhD 1982 Microbiology

Employment

1982 - 1984	Staff Fellow, LMB, DCBDC, NCI, NIH
1985 - 1987	Senior Staff Fellow, LMB, DCBDC, NCI, NIH
1987 - 1994	Microbiologist, LMB, DCBDC, NCI, NIH
1994-present	Chief, Biotherapy Section, LMB, DBS, NCI, NIH

Honors

January 1980	Awarded the Albert J. Ryan Fellowship.
June 1991	NIH Director's Award
June 1992	Pierce Immunotoxin Award, at The Third International Immunotoxin Meeting, Orlando, FL.
July 1994	Chair, Gordon Conference, *Drug Carriers in Medicine & Biology*.
September 1995	NIH Award of Merit
February 1999	Awarded NCI Intramural Research Award (IRA)
September 2004	NIH Merit Award
July 2002	NIAID Biodefense Grant Award

Teaching Experience

Was invited to teach a two-week (September - October, 1988) course on immunotoxins at the Shanghai Institute of Biochemistry (joint U.S. National Academy of Science and Chinese Academy of Science program).

Editorial Boards

Infection and Immunity (1987-1989)
Journal of National Cancer Institute (1990-1994)
Journal of Pharmaceutical Sciences
Journal of Bioconjugate Chemistry (1990-1994)
Journal of Drug Targeting
Therapeutic Immunity
Journal of Biological Chemistry (1996-)

Peer Review Experience

Member of Study Section for Tropical Medicine and Parasitology, October 1986
Member of special study section to review toxin-based grant proposals, July 1988
American Cancer Society, Ad Hoc Reviewer for Immunotherapy Study Section, Spring 1991

Clinical Investigation

Co-investigator on FDA-approved protocol with PE-ANTI-TAC to treat patients with adult-T-cell leukemia, IND #BB IND 2174 (NSC 600665).

Co-investigator on FDA-approved protocol with OVB3-PE to treat patients with ovarian cancer, IND #IND2688 (NSC 615048).

Co-investigator on FDA-approved protocol with LMB-1 to treat patients with adenocarcinomas IND #5017 (NSC 651311).

Co-investigator on Phase I application for IND of immunotoxin directed to CD22+ leukemias and lymphomas (IND/NSC numbers not yet available).

Committee Experience

An original member and presently serving on NCI's "Technology Review Group". Responsible for reviewing all new invention reports and making strategic decisions about how to prosecute NCI's existing patent portfolio.

Societies

AAAS
American Society for Biochemistry and Molecular Biology

Patents

Pastan, I., Willingham, M.C., and FitzGerald, D.J.: *Pseudomonas* exotoxin conjugate immunotoxins. (Assignee: U.S.A., D.H.H.S.) (Filed January 26, 1984.) Granted U.S. Patent #4,545,985, October 8, 1985.

Pastan, I., FitzGerald, D.J.P., and Willingham, M.C.: Monoclonal antibody against ovarian cancer cells (OVB3). Patent #4,806,494, February 21, 1989.

Pastan, I., Adhya, S., and FitzGerald, D.J.P.: Recombinant *Pseudomonas* exotoxin: Construction of an active immunotoxin with low side effects. Patent #4,892,827, January 9, 1990.

Bjorn, M.J., FitzGerald,D.J., Frankel, A.E.,Laird, W.J., Pastan, I.H., Ring, D.B., Willingham, M. C., and Windelhake, J. L.: Anti-human ovarian cancer immunotoxins and methods of use thereof. (Assignee; Cetus Corporation) (Filed July 6, 1987.) Granted U.S. Patent #4,958,009, September 18, 1990.

Pastan, I., FitzGerald, D., and Ogata, M.: Selectively cytotoxic IL-4-PE40 fusion protein. (Assignee: U.S.A., D.H.H.S.) (Filed May 12, 1989.) Granted U.S. Patent #5,082,927, January 21, 1992.

Berger, E.A., Fuerst, T.R., Pastan, I., FitzGerald, D., Mizukami, T., and Chaudhary, V.K.: CD-4/cytotoxic gene fusions. Patent #5,206,353, (Assignee: U.S.A., D.H.H.S.) (Filed July 22, 1988.) Granted U.S. Patent #5,206,353, April 27, 1993.

Pastan, I.H., Trevor, P., FitzGerald, D.J., Debinski, W., and Siegall, C.: Recombinant chimeric proteins deliverable across ellular membranes into cytosol of target cells. (Assignee: U.S.A., D.H.H.S.) (Filed March 4, 1991.) Granted U.S. Patent #5,328,984, July 12, 1994.

Berger, E.A., Moss, B., Fuerst, T.R., Pastan, I., FitzGerald, D., Mizukami, T., and Chaudhary, V.K.: Cytotoxic agent against specific virus infection. (Assignee: U.S.A.) (Filed February 25, 1993.) Granted U.S. Patent #5,428,143, June 27, 1995.

Pastan, I., Chaudhary, V.K., and FitzGerald, D.: P. exotoxin fusion proteins have COOH-terminal alterations which increase cytotoxicity. (Assignee: U.S.A., D.H.H.S.) (Filed May 14, 1990.) Granted U.S. Patent #5,458,878, October 17, 1995.

Pastan, I., FitzGerald, D., and Chaudhary, V.K.: Pseudomonas exotoxins (PE) and conjugates thereof having lower animal toxicity with high cytocidal activity through substitution of positively charged amino acids. (Assignee: U.S.A., D.H.H.S.) (Filed October 1, 1993.) Granted U.S. Patent #5,512,658, April 30, 1996.

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2. FitzGerald, D.J.P., Morris, R.E., and Saelinger, C.B.: The essential role of calcium in cellular internalization of *Pseudomonas* toxin. *Infect. Immun.* 35: 715-720, 1982.

3. FitzGerald, D.J.P., Padmanabhan, R., Pastan, I., and Willingham, M.C.: Adenovirus-induced release of epidermal growth factor and *Pseudomonas* toxin into the cytosol of KB cells during receptor-mediated endocytosis. *Cell* 32: 607-617, 1983.

4. FitzGerald, D.J.P., Trowbridge, I.S., Pastan, I., and Willingham, M.C.: Enhancement of toxicity of antitransferrin receptor antibody *Pseudomonas* exotoxin conjugates by adenovirus. *Proc. Natl. Acad. Sci. USA* 80: 4134-4138, 1983.

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9. Seth, P., FitzGerald, D.J.P., Willingham, M.C., and Pastan, I.: Role of a low pH environment in adenovirus enhancement of the toxicity of a *Pseudomonas* exotoxin epidermal growth factor conjugate. *J. Virol.* 51: 650-655, 1984.

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Pastrana DV, Fitzgerald DJ. A nonradioactive, cell-free method for measuring protein synthesis inhibition by *Pseudomonas* exotoxin. Anal Biochem. 2006 Apr 17; [Epub ahead of print]

EVIDENCE APPENDIX

PART II

EXHIBIT E2

1: Nature. 1988 Sep 22;335(6188):369-72.

nature

Selective killing of HIV-infected cells by recombinant human CD4-Pseudomonas exotoxin hybrid protein.

Chaudhary VK, Mizukami T, Fuerst TR, FitzGerald DJ, Moss B, Pastan I, Berger EA.

Laboratory of Molecular Biology, National Cancer Institute, Bethesda, Maryland 20892.

It is projected that in the absence of effective therapy, most individuals infected with human immunodeficiency virus (HIV) will develop acquired immune deficiency syndrome (AIDS) and ultimately succumb to a combination of opportunistic microbial infections, malignancies and direct pathogenic effects of the virus. Anti-viral agents, immunomodulators, and inhibitors of specific HIV functions are being tested as potential treatments to alleviate the high morbidity and mortality. An alternative therapeutic concept involves the development of cytotoxic agents that are targeted to kill HIV-infected cells. Here we describe the purification and characterization of a recombinant protein produced in Escherichia coli that contains the HIV-binding portion of the human CD4 molecule linked to active regions of Pseudomonas exotoxin A. This hybrid protein displays selective toxicity toward cells expressing the HIV envelope glycoprotein and thus represents a promising novel therapeutic agent for the treatment of AIDS.

PMID: 2843774 [PubMed - indexed for MEDLINE]

Exhibit 2

Oct 2 2006 08:20:36

EVIDENCE APPENDIX

PART II

EXHIBIT E3

1: AIDS Res Hum Retroviruses. 1990 Jun;6(6):795-804.

Recombinant CD4-Pseudomonas exotoxin hybrid protein displays HIV-specific cytotoxicity without affecting MHC class II-dependent functions.

Berger EA, **Chaudhary VK**, **Clouse KA**, **Jaraquemada D**, **Nicholas JA**, **Rubino KL**, **Fitzgerald DJ**, **Pastan I**, **Moss B**.

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892.

The present study describes several in vitro activities of CD4(178)-PE40, a recombinant protein containing a portion of human CD4 linked to active regions of Pseudomonas aeruginosa exotoxin A. Using assays for cell viability, we demonstrate that the hybrid toxin displays highly selective cytotoxicity for HIV-infected T lymphocytes. In a latently infected human T-cell line which is inducible for HIV expression, toxin sensitivity is observed only upon virus induction. At concentrations which readily kill HIV-infected T cells, CD4(178)-PE40 has no observable cytotoxic effects on uninfected human cell lines expressing surface major histocompatibility complex (MHC) Class II molecules, and does not interfere with cellular responses known to be dependent on functional association between CD4 and MHC Class II molecules.

PMID: 2114147 [PubMed - indexed for MEDLINE]

Exhibit 5

EVIDENCE APPENDIX

PART II

EXHIBIT E4

1: Proc Natl Acad Sci U S A. 1990 Nov;87(22):8889-93.

Elimination of infectious human immunodeficiency virus from human T-cell cultures by synergistic action of CD4-Pseudomonas exotoxin and reverse transcriptase inhibitors.

Ashorn P, Moss B, Weinstein JN, Chaudhary VK, FitzGerald DJ, Pastan I, Berger EA.

Laboratory of Viral Diseases, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892.

We have previously described a recombinant protein, designated CD4(178)-PE40, consisting of the human immunodeficiency virus (HIV) envelope glycoprotein-binding region of human CD4 linked to the translocation and ADP-ribosylation domains of Pseudomonas aeruginosa exotoxin A. By virtue of its affinity for gp120 (the external subunit of the HIV envelope glycoprotein), the hybrid toxin selectively binds to and kills HIV-1-infected human T cells expressing surface envelope glycoprotein and also inhibits HIV-1 spread in mixed cultures of infected and uninfected cells. We now report that CD4(178)-PE40 and reverse transcriptase inhibitors exert highly synergistic effects against HIV-1 spread in cultured human primary T cells. Furthermore, combination treatment can completely eliminate infectious HIV-1 from cultures of human T-cell lines. This conclusion is based on protection of a susceptible cell population from HIV-induced killing, complete inhibition of virus protein accumulation, and elimination of HIV DNA (as judged by quantitative polymerase chain reaction analysis). The results highlight the therapeutic potential of treatment regimens involving combination of a virostatic drug that inhibits virus replication plus an agent that selectively kills HIV-infected cells.

PMID: 1701055 [PubMed - indexed for MEDLINE]

Exhibit 4

EVIDENCE APPENDIX

PART II

EXHIBIT E5

1: Proc Natl Acad Sci U S A. 1989 Dec;86(23):9539-43.

CD4-Pseudomonas exotoxin hybrid protein blocks the spread of human immunodeficiency virus infection in vitro and is active against cells expressing the envelope glycoproteins from diverse primate immunodeficiency retroviruses.

Berger EA, Clouse KA, Chaudhary VK, Chakrabarti S, FitzGerald DJ, Pastan I, Moss B.

Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, MD 20852.

We previously described an unusual recombinant protein, designated CD4(178)-PE40, containing the gp120 binding region of human CD4 linked to active regions of Pseudomonas exotoxin A. The ability of this molecule to selectively inhibit protein synthesis in cells expressing the surface envelope glycoprotein of human immunodeficiency virus (HIV) suggested this molecule may be useful in treating infected individuals. To further evaluate its therapeutic potential, several in vitro properties of this hybrid toxin were examined. CD4(178)-PE40 was found to be an extremely potent cytotoxic agent, selectively killing HIV-infected cells with IC50 values around 100 pM. In a coculture system employing mixtures of HIV-infected and -uninfected cells, the hybrid toxin inhibited spread of the infection, as judged by a delay in HIV-induced cell killing and a dramatic suppression of free virus production. Experiments with control recombinant proteins indicated that this protective effect was primarily due to selective killing of the HIV-infected cells, rather than to a simple blocking effect of the CD4 moiety of the hybrid toxin. Using recombinant vaccinia viruses as expression vectors, we found the hybrid toxin to be active against cells expressing the envelope glycoproteins of divergent isolates of HIV-1, as well as HIV-2 and simian immunodeficiency virus. These results provide further support for the therapeutic potential of CD4(178)-PE40 in the treatment of HIV-infected individuals.

EVIDENCE APPENDIX

PART II

EXHIBIT E6

1: J Leukoc Biol. 2006 Aug 21; [Epub ahead of print]

Anti-HIV-1 immunotoxin 3B3(Fv)-PE38: enhanced potency against clinical isolates in human PMBCs and macrophages, and negligible hepatotoxicity in macaques.

Kennedy PE, Bera TK, Wang QC, Gallo M, Wagner W, Lewis MG, Berger EA, Pastan I.

*Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases and Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland; and Southern Research Institute, Frederick, Maryland.

Highly active antiretroviral therapy (HAART) against human immunodeficiency virus type 1 (HIV-1) infection dramatically suppresses viral load, leading to marked reductions in HIV-1 associated morbidity and mortality. However, infected cell reservoirs and low-level replication persist in the face of suppressive HAART, leading invariably to viral rebound upon cessation of treatment. Toxins engineered to target the Env glycoprotein on the surface of productively infected cells represent a complementary strategy to deplete these reservoirs. We described previously highly selective killing of Env-expressing cell lines by CD4(178)-PE40 and 3B3(Fv)-PE38, recombinant derivatives of Pseudomonas aeruginosa exotoxin A containing distinct targeting moieties against gp120. In the present report, we compare the in vitro potency and breadth of these chimeric toxins against multiple clinical HIV-1 isolates, replicating in biologically relevant primary human target cell types. In PBMCs, 3B3(Fv)-PE38 blocked spreading infection by all isolates examined, with greater potency than CD4(178)-PE40. 3B3(Fv)-PE38 also potently inhibited spreading HIV-1 infection in primary macrophages. Control experiments demonstrated that in both target cell types, most of the 3B3(Fv)-PE38 activity was due to selective killing of infected cells, and not merely to neutralization by the antibody moiety of the chimeric toxin. High-dose treatment of rhesus macaques with 3B3(Fv)-PE38 did not induce liver toxicity, whereas equivalent dosage of CD4(178)-PE40 induced mild hepatotoxicity. These findings highlight the potential use of 3B3(Fv)-PE38 for depleting HIV-infected cell reservoirs persisting in the face of HAART.

PMID: 16923920 [PubMed - as supplied by publisher]

Exhibit 6

EVIDENCE APPENDIX

PART II

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1: Bera TK, Kennedy PE, Berger EA, Barbas CF 3rd, Pastan I. Related Articles, Links

Specific killing of HIV-infected lymphocytes by a recombinant immunotoxin directed against the HIV-1 envelope glycoprotein.
Mol Med. 1998 Jun;4(6):384-91.
PMID: 10780881 [PubMed - indexed for MEDLINE]

2: Berger EA, Moss B, Pastan I. Related Articles, Links

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Proc Natl Acad Sci U S A. 1998 Sep 29;95(20):11511-3. Review. Erratum in: Proc Natl Acad Sci U S A 1999 Feb 2;96(3):1162.
PMID: 9751696 [PubMed - indexed for MEDLINE]

3: Martin-Serrano J, Folgueira L, Lain de Lera T, Pedraza MA, Lemichez E, Sanchez-Palomino S, Noriega AR, Boquet P, Alcami J. Related Articles, Links

In vitro selective elimination of HIV-infected cells from peripheral blood in AIDS patients by the immunotoxin DAB389CD4.
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4: Van Oijen MG, Preijers FW. Related Articles, Links

Rationale for the use of immunotoxins in the treatment of HIV-infected humans.
J Drug Target. 1998;5(2):75-91. Review.
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Immunologic cross-reaction between HIV type 1 p17 and Mycoplasma hyorhinis variable lipoprotein.
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Therapeutic potential of anti-HIV immunotoxins.
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In vitro effects of anti-HIV immunotoxins directed against multiple epitopes on HIV type 1 envelope glycoprotein 160.
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☐ **9:** Borvak J, Chou CS, Bell K, Van Dyke G, Zola H, Ramilo O, Vitetta ES. Related Articles, Links

Expression of CD25 defines peripheral blood mononuclear cells with productive versus latent HIV infection.
J Immunol. 1995 Sep 15;155(6):3196-204.
PMID: 7673732 [PubMed - indexed for MEDLINE]

☐ **10:** Davey RT Jr, Boenning CM, Herpin BR, Batts DH, Metcalf JA, Wathen L, Cox SR, Polis MA, Kovacs JA, Falloon J, et al. Related Articles, Links

Use of recombinant soluble CD4 Pseudomonas exotoxin, a novel immunotoxin, for treatment of persons infected with human immunodeficiency virus.
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J Immunol. 1993 Jun 1;150(11):5202-8.
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☐ **13:** Pincus SH, Messer KG, Schwartz DH, Lewis GK, Graham BS, Blattner WA, Fisher G. Related Articles, Links

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J Clin Invest. 1993 May;91(5):1987-96.
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☐ **14:** Erice A, Balfour HH Jr, Myers DE, Leske VL, Sannerud KJ, Kuebelbeck V, Irvin JD, Uckun FM. Related Articles, Links

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Items 1 - 20 of 26 Page 1 of 2 Next

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Items 21 - 26 of 26 Previous Page 2 of 2

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